[Chem. Pharm.Bull. 15(2) 159～163 (1967)]

UDC 581.19 : 581.143 : 582.736 : 547.757

21. Toshihiko Okamoto,*1 Yo Isogai,*2 and Tôru Koizumi*1 : Studies on Plant Growth Regulators. II.*3 Isolation of Indole-3-acetic Acid, Phenylacetic Acid, and Several Plant Growth Inhibitors from Etiolated Seedlings of Phaseolus.

(*Faculty of Pharmaceutical Sciences, University of Tokyo*2 and Biological Institute, College of General Education, University of Tokyo*3*)

Methyl indoleacetate and methyl phenylacetate were isolated from the methylated F-2 fraction of Moyashi aqueous extract. And three methylated plant growth inhibitors were also isolated.

(Received April 27, 1966)

One of the present authors (Y. Isogai) had reported the both neutral (F-1) and acidic (F-2) fractions obtained from aqueous extract of Moyashi had as a whole growth promoting action on oat coleoptile sections. He further reported that the F-1 fraction contained only growth promotors while the F-2 fraction contained both growth promotor (having the same Rf value of IAA) and inhibitor by paper chromatography.[1)]

The treatment of the F-1 fraction had already been reported in the previous paper, now we studied the F-2 fraction and isolated several growth regulators. The method and the results will be described in this paper.

The method of extraction, the properties of the F-2 fraction, and the yield of F-2 fraction in the large scale extraction have been reported in the preceding paper.[2)] In the present work, adsorption chromatography with alumina was mainly employed, therefore, the F-2 fraction was methylated with diazomethane and methylated F-2 fraction (Me-F-2) was separated by column chromatography. From a total of 68.5 g. of F-2 fraction obtained in the II～VIII large scale extractions several small samples were methylated by diazomethane in MeOH and were tested for recovery of activity in the eluate of the column chromatography on alumina. This was shown in Fig. 1. Each fraction was hydrolyzed to acidic products and tested by the Avena straight growth test.

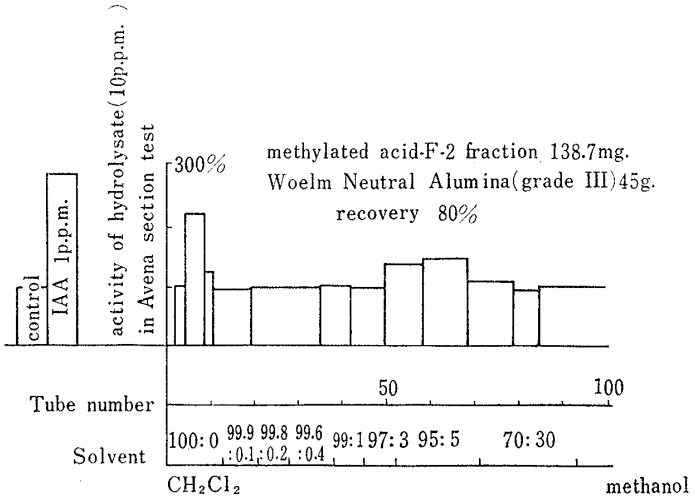

Fig. 1. Chromatography of Methylated Acid-F-2 Fraction

*1 Hongo, Tokyo (岡本敏彦, 小泉 徹).
*2 Komaba-cho, Meguro-ku, Tokyo (磯谷 遙).
*3 This paper constitutes a part of series entitled "Studies on Plant Growth Regulators" by Y. Isogai, T. Okamoto, and T. Koizumi. Part I: This Bulletin, **15**, 151 (1967).

1) Y. Isogai: Sci. Pap. Coll. Gen. Educ., Univ. Tokyo, **10**, 73 (1960).
2) Part I: This Bulletin, **15**, 151 (1967).

Since the recovery of activity seemed to be nearly complete, the chromatographic procedure were carried out for the methylated F-2 fraction. 45 g. of Me-F-2 was subjected to alumina column chromatography with Woelm neutral alumina (Wn alumina). This was shown in Fig. 2.

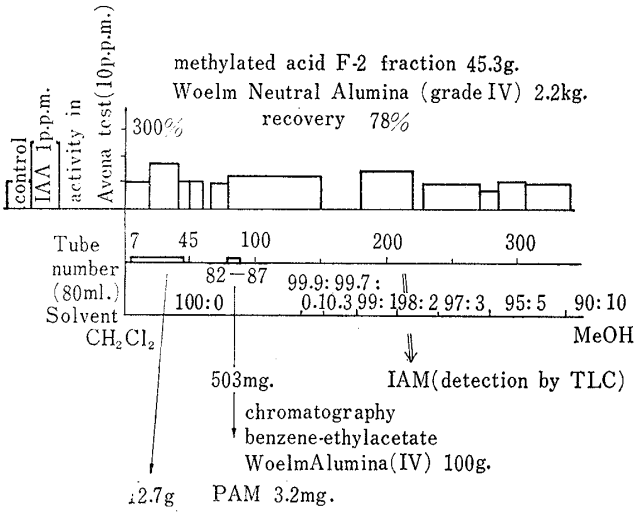

Fig. 2. Chromatography of Methylated Acid-F-2 Fraction

The eluates from Fr. 82~200 gave a weak growth promoting activity and several growth promotors seemed to be contained in this fraction. In order to isolate these growth promotors, the deposit from Fr. 82~87 was rechromatographed and 3.2 mg. of PAM was obtained. The residue from Fr. 90~200 was further studied and the presence of IAM was confirmed by the thin-layer chromatography. Thus, it was concluded that the acidic F-2 fraction was contaminated with phenylacetamide and indole-3-acetamide in the F-1 fraction. The deposit from Fr. 7~45 had also growth promoting activity and was subjected to adsorption column chromatography with silica gel. This was shown in Fig. 3. The eluate from Fr. 71~78 contained methyl indoleacetate (Me-IAA) as detected by the thin-layer chromatography and was rechromatographed, yielding 47.8 mg. of pure methyl indoleacetate and this was hydrolyzed to indole-3-acetic acid. Methyl indoleacetate was also detected in the methylated F-2 fraction by gas chromatography. The deposit from Fr. 31~34 and the deposit from Fr. 150~190 (Fig. 3) gave weak growth inhibition when they were tested by Avena straight growth test in their methylated form without alkaline hydrolysis.

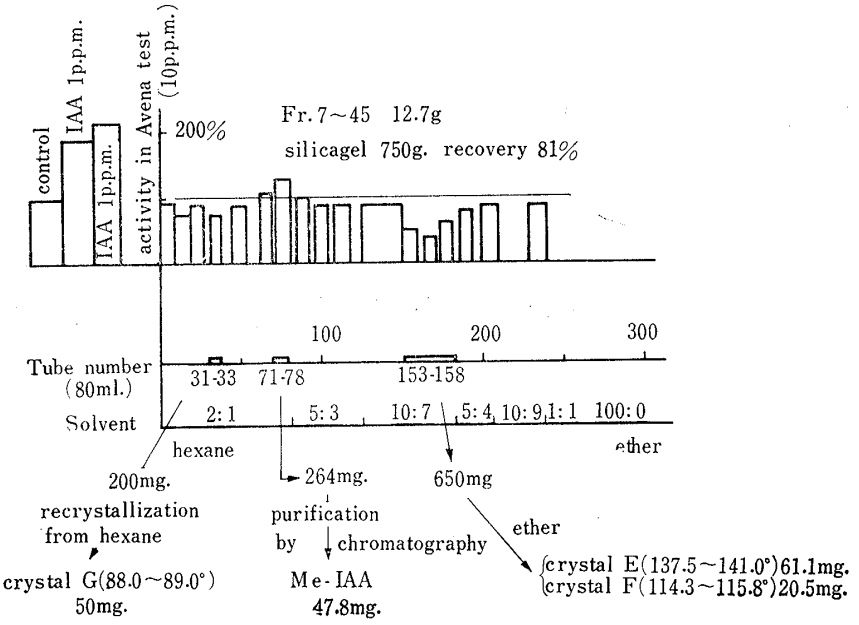

Fig. 3. Chromatography of Fr. 7~45

And two pure substances were obtained from Fr. 150~190 (which was tentatively called as crystal E and F) and another crystalline substance was obtained from Fr. 31~34. (It was tentatively designated as crystal G.)

These three crystals have weak growth inhibitory activity. However they are the methylated form of natural plant growth inhibitors, so the structure determination of these substances and the study of the inhibitory action of natural (demethylated) growth inhibitors are quite important. The structure determination are now in progress and the action of natural growth inhibitors will be reported in the near future.

Since methyl phenylacetate (Me-PAA) was expected to be adsorbed in the front zone of the column (Fig. 3), each fraction corresponding to the zone was studied by gas chromatography. Me-PAA was detected in the Fr. 23~24.

Experimental

Bioassay Method (see Part I)——Any special assay methods were not employed to detect the growth inhibitors. In the Avena straight growth test which we have used, the control sections floated on water for 24 hr. also gave considerable amount of elongation. In this paper, any substance which gave shorter coleoptile sections as compared to the final length of control ones was regarded as growth inhibitor.

Material——The acidic F-2 fraction (68.59) of Moyashi aqueous extract which was obtained by the large scale extraction (see Part I) was used.

Methylation of F-2 Fraction——The oily F-2 fraction was dissolved in small quantity of MeOH and the diazomethane etherate was added in this methanolic solution dropwise. After the evolution of gas stopped, an additional few drops of diazomethane etherate was added and kept overnight in the refrigerator. The solvent was evaporated *in vacuo* and yellow methylated F-2 fraction was obtained.

Column Chromatography 1) Chromatography of Me-F-2 (Fig. 1)——138.7 mg. of Me-F-2 was chromatographed with 45 g. of Wn alumina (activity grade Ⅲ) (1 Fr. 15 ml.). Each eluate was evaporated and hydrolyzed with *N*-KOH in MeOH for 1 hr. and subjected to bioassay. Recovery and eluting solvent system are as follows.

Fraction No.	Solvent system	
1~12	CH_2Cl_2	
13~20	CH_2Cl_2–MeOH (99.9:0.1)	
21~27	CH_2Cl_2–MeOH (99.8:0.2)	
28~37	CH_2Cl_2–MeOH (99.6:0.4)	
38~44	CH_2Cl_2–MeOH (99:1)	
45~51	CH_2Cl_2–MeOH (97:3)	
52~70	CH_2Cl_2–MeOH (95:5)	
71~92	CH_2Cl_2–MeOH (70:30)	
93~	MeOH	Recovery 80%

2) **Large Scale Chromatography** (Fig. 2)——45.3 g. of Me-F-2 was chromatographed with 2.12 kg. of Wn alumina (grade Ⅳ). Recovery and eluting solvent system were as follows (1 Fr. 80 ml.).

Fraction No.	Solvent system	
1~134	CH_2Cl_2	
135~151	CH_2Cl_2–MeOH (99.9:0.1)	
152~177	CH_2Cl_2–MeOH (99.7:0.3)	
178~208	CH_2Cl_2–MeOH (99:1)	
209~238	CH_2Cl_2–MeOH (98:2)	
239~278	CH_2Cl_2–MeOH (97:3)	
279~334	CH_2Cl_2–MeOH (95:5)	
335~341	CH_2Cl_2–MeOH (90:10)	
342~	MeOH	Recovery 78%

The eluate from Fr. 82~87 yielded 503 mg. of amorphous solid and gave growth promoting activity. The eluate from Fr. 279~335 yielded 4.9 g. of amorphous solid but this was inactive.

3) **Rechromatography of Fr. 82~87**——503 mg. of residue obtained from Fr. 82~87 was chromatographed with 100 g. of Wn alumina (grade Ⅳ) (1 Fr. 15 ml.).

Fraction No.	Solvent system	
1~ 23	benzene–AcOEt (20:1)	
24~ 46	benzene–AcOEt (100:7)	
47~ 86	benzene–AcOEt (100:10)	
87~111	benzene–AcOEt (100:15)	Recovery 91%

54 mg. of amorphous solid was obtained from the eluate of Fr. 95~113 and was recrystallized from Et_2O yielding 3.2 mg. of pure sample (m.p. 156°). It is identified as phenylacetamide by its melting point

and IR spectrum and was confirmed by admixture. The growth promoting activity of this fraction was attributed to PAM.

Gas Chromatography of Me-F-2——Me-F-2 and Me-IAA were dissolved in ether and subjected to gas chromatography using Ohkura's Gas Chromatograph (flame ionization type detector). Composition and temperature of the column used and other conditions were as follows.

Column : 5% DC 550 on Chromosorb W (60～90 mesh) 6.3 mm. × 2 m.
Column temperature : 220°
Evaporation temp. : 330°
Carrier gas : He 1 kg./cm²

Me-IAA has a peak at a retention time of 27.5 min. and Me-F-2 also has a small peak (shoulder) at retention time of 27.5 min. It was concluded that Me-IAA was included in the Me-F-2 fraction though quite a little amount.

Detection of IAM in Fr. 90～200 by TLC——In order to detect IAM in the Fr. 90～200, these fractions were analyzed by TLC. TLC was performed on Kieselgel G (Fluka Co.) using Ehrlich reagent as a spray reagent. IAM was detected in the following solvent system in the Fr. 90～200.

AcOEt Rf 0.1
Me_2CO Rf 0.4

4) **Rechromatography of Fr. 7～45 (Fig. 3)**——12.7 g. of the deposit obtained from Fr. 7～45 in large scale chromatography was rechromatographed with 750 g. of silica gel (Kanto Kagaku Co.) (1 Fr. 80 ml.).

Fraction No.	Solvent system
1～ 81	*n*-hexane-ether (2:1)
82～125	*n*-hexane-ether (5:3)
126～182	*n*-hexane-ether (10:7)
183～206	*n*-hexane-ether (10:8)
207～238	*n*-hexane-ether (10:9)
239～257	*n*-hexane-ether (1:1)
258～	ether

The eluate from Fr. 31～34 gave growth inhibition and yielded 200 mg. of amorphous solid. This amorphous solid was recrystallized from *n*-hexane yielding 50 mg. of pure sample (m.p. 88.0～89.0°) which was tentatively designated as crystal G. The eluate from Fr. 150～190 also gave growth inhibition and yielded 650 mg. of colorless crystal. This was recrystallized from ether yielding two kinds of substances, 66 mg. of crystal E (m.p. 137.5～141.0°) and 20.5 mg. of crystal F (114.3～115.8°). The pure samples of these three crystals gave weak growth inhibition. 204 mg. of the residue obtained in Fr. 71～78 contained Me-IAA as determined by TLC (Tosinlayer G ether-hexane 1:1 Rf 0.2) and was rechromatographed.

Rechromatography of Fr. 71～78 and Isolation of Me-IAA——204 mg. of the residue from Fr. 71～78 was rechromatographed with 52 g. of silica gel using the mixture of hexane and ether (as eluting solvent. 179 mg. of the deposit eluted with *n*-hexane-ether (5:3) contained Me-IAA and it was rechromatographed with 50 g. of Wn alumina (grade Ⅳ) using benzene as eluting solvent. 47.8 mg. of the oily matter obtained in Fr. 7～14 was pure Me-IAA and it was confirmed by comparison of the IR spectrum with that of authentic Me-IAA.

Hydrolysis of Isolated Me-IAA——A mixture of 26 mg. of isolated Me-IAA and 40 mg. of NaOH and 5 ml. of H_2O and 5 ml. of MeOH was refluxed for 2 hr. After cooling, the reaction mixture was acidified with conc. HCl to give crystalline precipitate. Recrystallization from $CHCl_3$ gave 16 mg. of indole-3-acetic acid (m.p. 162～163°). It was confirmed by the mixed melting point and by comparing the IR spectrum with that of authentic specimen.

The Detection of Me-PAA by Gas Chromatography——The oily material obtained from Fr. 23～24 (see Fig. 3) was subjected to gas chromatography using Ohkura's Gas Chromatograph (flame ionization type detector). Composition and temperature of the column used and other conditions were as follows.

Column : DC 550 on Chromosorb W (60～90 mesh) 6.3 mm. × 2 m.
Column temperature : 165°
Evaporation temp. : 200°
Carrier gas : N_2 0.9 kg./cm²

Me-PAA has a peak at a retention time of 3.7 min. and Me-F-2 has a small peak at retention time of 3.7 min. It was concluded that Me-PAA was contained in the Me-F-2 though quite a little amount.

The authors wish to express their appreciation to Mr. M. Wakaomi of the Yûki Gôsei Kôgyo Co., Ltd., and to Mr. H. Yamaguchi of the Yawata Chemical Engineering Co., Ltd. for the kind aid they have given in carrying out the large scale extraction.

This research was supported in part by Grant-in-Aid for Developmental Scientific Research from the Ministry of Education for which the authors wish to express their gratitude. They are also grateful to the members of Central Analysis Room of the Faculty for elemental analysis and spectral data.

[Chem. Pharm. Bull. 15(2) 163～168 (1967)]

UDC 581.19 : 581.143 : 582.736 : 547.757

22. Toshihiko Okamoto,*1 Yo Isogai,*2 Tôru Koizumi,*1 Hisako Fujishiro,*1 and Yaeko Sato*2 : Studies on Plant Growth Regulators. III.*3 Isolation of Indole-3-acetonitrile and Methyl Indole-3-acetate from the Neutral Fraction of the Moyashi Extract.

(*Faculty of Pharmaceutical Sciences, University of Tokyo,*1 and Biological Institute, College of General Education, University of Tokyo*3)

Methyl indole-3-acetate and indole-3-acetonitrile were identified in the neutral fraction of Moyashi (F-1 AcOEt fraction) by thin-layer chromatography and vapor phase chromatography.

(Received April 27, 1966)

In the previous paper[1] it was reported that indole-3-acetamide and phenylacetamide were isolated from the neutral fraction (F-1) of Moyashi by alumina column chromatography using a mixture of ethylacetate and methanol as the eluting solvent. It was also reported that the growth promoting activity was found in the ethyl acetate eluting fraction by the alumina chromatography.[1] (It was tentatively called as F-1 AcOEt fraction.)

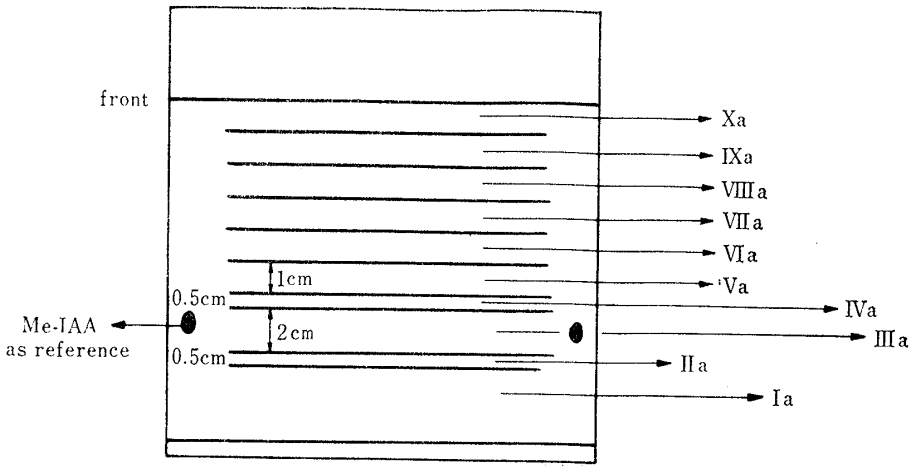

Fig. 1. Preliminary Thin-layer Chromatography
adsorbent : Camag Kieselgel
solvent : Ether-Hexane (2:1)

*1 Hongo, Tokyo (岡本敏彦，小泉　徹，藤代尚子).
*2 Komaba-cho, Meguro-ku, Tokyo (磯谷　遙，佐藤八重子).
*3 Part II. T. Okamoto, Y. Isogai, T. Koizumi : This Bulletin, **15**, 159 (1967).
1) Y. Isogai, T. Okamoto, T. Koizumi : This Bulletin, **15**, 151 (1967).