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CHARACTERIZATION OF GIBBERELLINS FROM LIGHT-GROWN *PHASEOLUS COCCINEUS* SEEDLINGS BY COMBINED GC–MS

D. H. BOWEN*
School of Chemistry, University of Bristol, Bristol

A. CROZIER†
Botany Department, University of Canterbury, Christchurch, New Zealand

J. MACMILLAN‡
School of Chemistry, University of Bristol, Bristol

D. M. REID§
Biology Department, University of Calgary, Calgary, Alberta, Canada

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Key Word Index—*Phaseolus coccineus*; Leguminosae; gibberellins A_1, A_4, A_5 and A_{20}; combined GC–MS.

Abstract—Following extensive purification of an extract from 5000 light-grown *Phaseolus coccineus* seedlings by procedures including countercurrent distribution, Sephadex G10, polyvinylpyrrolidone, charcoal–celite and silicic acid partition column chromatography, TLC and preparative GLC, gibberellins A_1, A_4, A_5 and A_{20} were characterized by combined GC–MS. In addition, an unknown compound isomeric with gibberellin A_{17} but lacking an hydroxyl group, was also detected.

INTRODUCTION

Phaseolus coccineus L. tissues contain a number of gibberellins (GAs). Use of GLC and combined GC–MS has facilitated the identification of GA_1, GA_5, GA_6, GA_8, GA_{17}, GA_{19} and GA_{20} in extracts from immature seed.[1,2] In addition GA_3, GA_8-glucoside[3] and GA_4[4] have been detected in *P. coccineus* seeds. *P. coccineus* seedlings appear to contain similar GAs since after silicic acid partition column chromatography of a partially purified extract from etiolated seedlings, GA_4 was characterized by GC–MS and GA_1 and GA_{19} were tentatively identified on GLC and bioassay data.[5]

This paper reports on an investigation into the endogenous GAs of light-grown *P. coccineus* seedlings.

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† Supported by grants from the Nuffield Foundation (New Zealand) and the University of Canterbury. Present address: Botany Department, University of Glasgow, Glasgow G12 8QQ, Scotland.
‡ Supported by NATO grant.
§ Supported by National Research Council (Canada) grant A-5727.

[1] MACMILLAN, J. and PRYCE, R. J. (1968) *Soc. Chem. Ind. Monogr.* **31**, 36.
[2] DURLEY, R. C., MACMILLAN, J. and PRYCE, R. J. (1971) *Phytochemistry* **10**, 1891.
[3] SEMBDNER, G., WEILAND, J., AURICH, O. and SCHREIBER, K. (1968) *Soc. Chem. Ind. Monogr.* **31**, 70.
[4] SEMBDNER, G. and SCHREIBER, K. unpublished data (see Refs. 5 and 7).
[5] CROZIER, A., BOWEN, D. H., MACMILLAN, J., REID, D. M. and MOST, B. H. (1971) *Planta* **97**, 142.

RESULTS

The acidic, ethyl acetate-soluble fraction from 4·5 kg of lyophilized, light-grown seedlings of *P. coccineus* was purified by slurrying with PVP, followed by CCD and successive column chromatography on Sephadex G10, PVP and charcoal–celite columns. The resultant partially purified fraction of 301 mg was re-chromatographed on a charcoal–celite column, eluted with a modified gradient of increasing amounts of acetone in water. Fractions (43) were collected and tested for biological activity in the dwarf rice bioassay. It was found that GA-like activity, totalling 30–40 μg of GA_3 equivalents was distributed between fractions 10–38. Fractions 10–38 were combined and the extract (201 mg) chromatographed on a silicic acid partition column. The 25 fractions from this column were tested for biological activity in three bioassays. The results are shown in Fig. 1. Although the dry weights of the fractions were still high, by considering the elution pattern of the column and the biological activities of the fractions in the bioassays, tentative assignments for some of the GAs present were made at this stage (Fig. 1). Further purification of the separated silicic acid column fractions 5, 8/9, 11 and 13 by elution from individual charcoal–celite columns increased the specific biological activity (Fig. 2, Table 1). GLC examination of the methyl esters of the most active fractions from the charcoal–celite columns showed peaks which chromatographed like some of the GAs thought to be present from the first tentative assignment (Table 1). Attempts to identify the GA-like peaks by GC–MS were unsuccessful due to the high concentrations of other endogenous plant material. TLC and preparative GLC of the methyl esters of the active fractions produced samples which were sufficiently pure to enable GC–MS to be carried out. GC–MS of the methyl esters and the trimethylsilyl ethers of the methyl esters of the GA-like peaks produced MS which could, except in one case, be correlated with those of known GAs.[6]

TABLE 1. TENTATIVE ASSIGNMENT OF GAS FOLLOWING STEPWISE ELUTION FROM CHARCOAL–CELITE COLUMNS WITH AQUEOUS ACETONE AND GLC OF METHYL ESTERS ON 2% SE33 AT 210°

Fraction	% Acetone	Wt (mg)	Gibberellin
5–2	65	2·3	A_4
8/9–1	50	3·7	A_5, A_{20}
8/9–2	60	5·1	A_5, A_{20}
11–6	60	2·2	A_{19}
13–2	50	3·1	A_1

TABLE 2. CHARACTERIZATION OF GAS BY GC–MS FOLLOWING PURIFICATION BY TLC AND PREPARATIVE GLC

Fraction	Scan No.	Gibberellin
5–2	6	A_4
8/9–1	3	A_5, A_{20}
11–6	5	Unknown
13–2	5	A_1

Scan No's. refer to scans indicated in Fig. 4

The logarithmic trace of the total ion current obtained from the preparative GLC purified trimethylsilyl ethers of the methyl esters of fractions 5–2, 8/9–1, 11–6 and 13–2 are shown in Fig. 3. It can be seen from these traces that the level of impurities in these fractions was still very high. Nevertheless, the MS obtained allowed unequivocal identification of the GAs found in fractions 5–2, 8/9–1 and 13–2. The gibberellins identified are shown in Table 2 together with the scan number from Fig. 3.

[6] BINKS, R., MACMILLAN, J. and PRYCE, R. J. (1969) *Phytochemistry* **8**, 271.

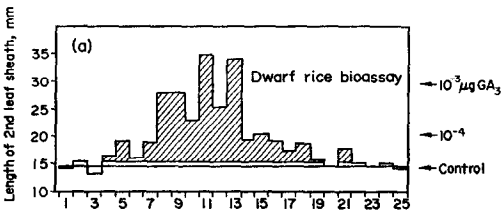

FIG. 1(a).

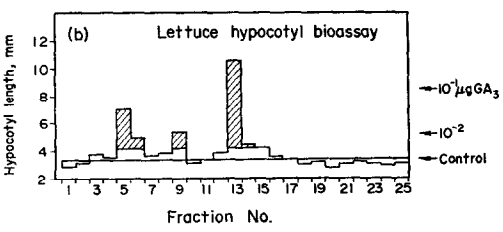

FIG. 1(b).

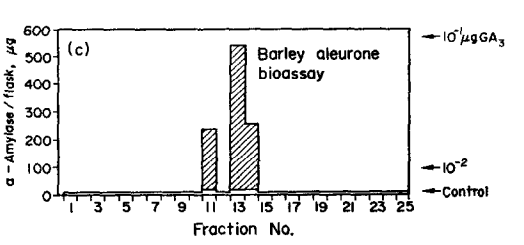

FIG. 1(c).

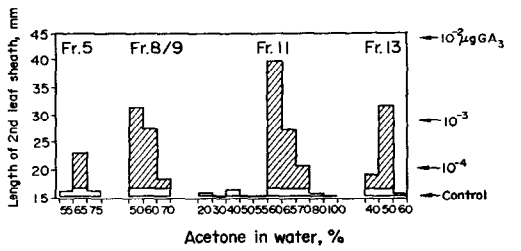

FIG. 2.

FIG. 1. DISTRIBUTION OF GA-LIKE ACTIVITY FOLLOWING SILICIC ACID PARTITION COLUMN CHROMATOGRAPHY.

Fractions tested for GA-like activity in the dwarf rice bioassay at a 1000-fold dilution, the lettuce hypocotyl bioassay at a 100-fold dilution, and the barley aleurone bioassay at a 200-fold dilution. Shaded areas of the histograms indicate stimulation above controls that is significant at the 5% level. The fractions examined further and their weight and tentative assignments are: 5, 13·2 mg, GA_4: 8/9, 22·2 mg, GA_5, GA_6 and GA_{20}; 11, 12·5 mg, GA_{19}; 13, 19·2 mg GA_1 and GA_3.

FIG. 2. DWARF RICE BIOASSAYS OF SILICIC ACID COLUMN FRACTIONS 5, 8/9, 11 AND 13 FOLLOWING STEPWISE ELUTION FROM CHARCOAL–CELITE COLUMNS WITH AQUEOUS ACETONE

Eluates from the fraction 5 column tested at a 200-fold dilution; all other eluates tested at a 500-fold dilution.

Scan 5 of the trimethylsilyl ether of the methyl ester of *fraction* 11–6 produced a spectrum which had no ions at *m/e* 73 and 75. The line diagram of the methyl ester showed there is no ion at M^+-18. Clearly there is no hydroxyl group in this compound but in all other respects it is isomeric with GA_{17}. An ion at *m/e* 149 is almost certainly due to phthalate plasticiser impurity.

DISCUSSION

Following extensive purification procedures GC–MS has been used to characterize GA_1, GA_4, GA_5 and GA_{20} in extracts from light-grown *P. coccineus* seedlings. GA_4 eluted from a silicic acid partition column chromatogram in fraction 5, GA_5 and GA_{20} in fractions 8/9 and GA_1 in fraction 13. In addition, an as yet uncharacterized GA, isomeric with GA_{17} but containing no hydroxyl group, was detected in silicic acid column fraction 11. The data indicate that there are distinct similarities in the endogenous GAs of immature seeds and light-grown seedlings as GA_1, GA_4, GA_5 and GA_{20} have all been detected in extracts from immature *P. coccineus* seed.[7] However, the levels of GAs in light-grown seedlings are of a much lower order of magnitude than those found in immature seed.

[7] MACMILLAN, J. (1972) in *Plant Growth Substances* 1970 (CARR, D. J., ed.), p. 790, Springer, Berlin.

A comparison of the silicic acid column-bioassay data presented in this paper (Fig. 1) and equivalent data published by Crozier *et al.*[5] shows that there are also similarities in the GA content of light- and dark-grown *P. coccineus* seedlings. A precise, detailed quantitative and qualitative comparison of the endogenous GAs in the two tissues is not possible because not all the GA-like compounds have been characterized and the purification procedures employed by Crozier *et al.*[5] were somewhat simpler than those used in the current investigation. However, even allowing for greater losses in the extensive purification procedures of the present work, there can be little doubt that, overall, light-grown seedlings contain lower amounts of GA than their etiolated counterparts. With regard to individual GAs, there is a sharp contrast in the levels of GA_4. GA_4 constitutes a main GA in dark-grown seedlings[5] while in light-grown material it is a relatively minor component and

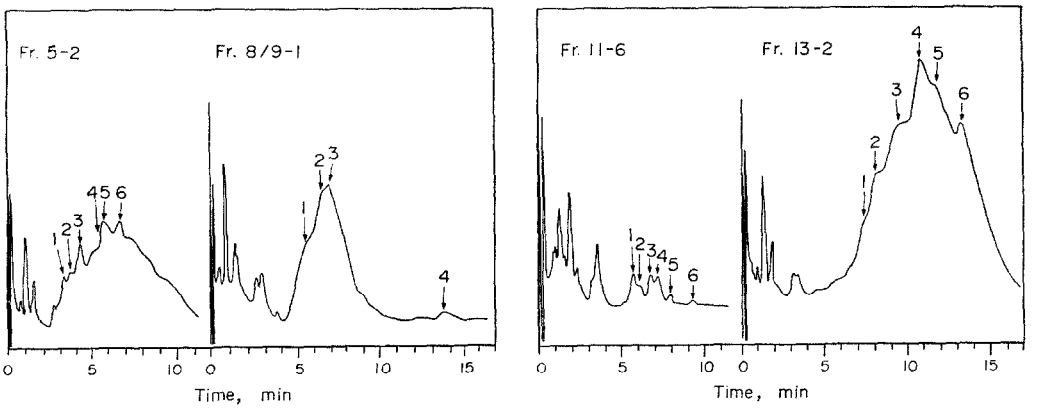

FIG. 3. LOGARITHMETIC TRACES OF TOTAL ION CURRENT OF THE TRI-METHYLSILYL ETHER OF THE METHYL ESTER OF PURIFIED FRACTIONS 5–2, 8/9–1 AND 13–2 ON A 2% SE33 COLUMN.
The trimethylsilyl ether of the methyl ester of purified fraction 11–6 was chromatographed on a 2% QF1 column. Columns programmed from 200 to 230° at 2°/min.

present in much lower quantities than GA_1, GA_5 and GA_{20} (Fig. 1). It would therefore appear that light may have some effect on GA metabolism which results in the GA_4 pool size being disproportionately reduced. However, in the past 18 months a number of purified extracts from etiolated *P. coccineus* seedlings have been analysed, using silicic acid column chromatography in conjunction with lettuce and dwarf rice bioassays, and without exception GA_4-like activity was present in only relatively small amounts.[8,9] In all instances the seedlings were grown in conditions identical to those used by Crozier *et al.*[5] It is therefore unlikely that environmental influences could have such a pronounced effect on the endogenous level of GA_4. In this connection, it is relevant to note that although GA_4 has been isolated from *P. coccineus* seed by Schneider and Sembdner,[4] neither MacMillan and Pryce[1] nor Durley *et al.*[2] detected GA_4 in extracts from apparently similar seed. A possible explanation for these differences in GA_4 levels in seed and seedlings may be that *P. coccineus* cv. Prizewinner is not a pure strain and that commercial supplies of the seed vary genetically.

Identification of GAs by chromatographic methods is only circumstantial.[10,11] The

[8] CROZIER, A. and REEVE, D. R. unpublished data.
[9] LANCASTER, J. (1972) B.Sc. Thesis, University of Canterbury.

total ion current traces illustrated in Fig. 3 show that even after most extensive purification the extracts were still grossly impure. In view of the fact that only four of the scans produced MS identifiable as GAs (Table 2), it would seem that the designation of GLC peaks as GAs solely on the basis of retention times is foolhardy. This is particularly so when only small amounts of tissue are extracted and the purification procedures used are rudimentary. Consequently the assignment of GA numbers to GLC peaks by Aung *et al.*,[12] Jeffcoat and Cockshull,[13] Perez and Lachman[14] and Ross and Bradbeer[15,16] should be viewed with some degree of skepticism in the absence of more conclusive data. In our previous paper on the GAs in dark-grown *P. coccineus* seedlings we reported the characterization of GA_4 by GC–MS, and on the basis of chromatographic and bioassay data tentatively identified GA_1 and GA_{19}.[5] Subsequent attempts to establish their identity by GC–MS were precluded by the loss of the samples in a laboratory fire. From the data of Durley *et al.*[17] indicating that GA_{19} is eluted from a silicic acid partition column in fraction 11 and the data presented in this paper, it seems possible that the biological activity in fraction 8 that was tentatively labelled as GA_{19} may well be GA_5 and GA_{20}. The identification of GA_1 in etiolated seedlings was, in all probability, correct.

The fact that silicic acid column fraction 8 from extracts of light-grown *P. coccineus* seedlings contains GA_5 and GA_{20}, and not GA_{19}, has some bearing on the proposal of Crozier and Reid[18,19] that root apices could be a site for the conversion of GA_{19} to GA_1. Crozier and Reid's data may now imply that root apices are a site for the synthesis of GA_1 from GA_5 and/or GA_{20}. This possibility is currently under investigation.

EXPERIMENTAL

Plant material. Seeds of *Phaseolus coccineus* cv. Prizewinner* were soaked in aerated running tap water at *ca.* 25° for 24 hr before being placed between sheets of filter paper and stored in darkness at 25° for 72 hr. After this period seeds with adequate root growth were transferred to aerated water culture and grown at 25° under a natural photoperiod of 12–13 hr for 8 days. *ca.* 5000 seedlings, 37·4 kg fr. wt, were frozen with liq. N_2 and lyophilized before extraction.

Extraction, purification and chromatography. The lyophilized tissue (4·5 kg) was macerated and extracted 3× with cold 70% MeOH (1 l./100 g). The combined methanolic extracts were reduced to the aqueous phase *in vacuo*. An equal vol. of 1·0 M, pH 8·0 phosphate buffer was added and the pH of extract adjusted to 8·0 with KOH. This was partitioned with 1/3 vol. light petrol. (b.p. 60–80°) until no more pigment was removed. The aqueous phase was then partitioned 8× with 2/5 vol. EtOAc. The EtOAc phase was dried (Na_2SO_4), reduced to about 50 ml *in vacuo* and partitioned 5× against equal vols of 1·0 M, pH 8·0 phosphate buffer. The EtOAc phase was discarded. The buffer was brought to pH 2·5 and partitioned 2× with 2/5 vol. EtOAc which was dried (EtOAc 1). The original aqueous phase was taken to pH with 2·5 H_2SO_4 and partitioned 5× with 2/5 vol. EtOAc. This phase was combined with EtOAc 1 and taken to dryness *in vacuo* giving the acidic, ethyl acetate soluble fraction.

The acidic, ethyl acetate-soluble fraction (29·5 g gum) was slurried with polyvinylpyrrolidone (PVP) in 0·2 M phosphate buffer.[20] The semi-purified acidic, ethyl acetate-soluble fraction (17·5 g) was subjected

* Purchased from Messrs. Carter s Tested Seeds Ltd., Raynes Park, London, SW 20, England.

[10] MacMillan, J. (1968) in *Biochemistry and Physiology of Plant Growth Substances* (Wightman, F. and Setterfield, G., eds.), p. 101, Runge Press, Ottawa.
[11] MacMillan, J. (1972) in *Hormonal Regulation of Plant Growth and Development* (Kaldeway, H. and Vardar, Y., eds.), p. 175, Chemie, Weinheim.
[12] Aung, L. H., de Hertogh, A. A. and Stably, G. L. (1971) *Phytochemistry* **10**, 215.
[13] Jeffcoat, B. and Cockshull, K. E. (1972) *J. Exp. Bot.* **23**, 722.
[14] Perez, A. T. and Lachman, W. H. (1971) *Phytochemistry* **10**, 2799.
[15] Ross, J. D. and Bradbeer, J. W. (1971) *Planta* **100**, 288.
[16] Ross, J. D. and Bradbeer, J. W. (1971) *Planta* **100**, 303.
[17] Durley, R. C., Crozier, A., Pharis, R. P. and McLaughlin, G. E. (1972) *Phytochemistry* **11**, 3029.
[18] Crozier, A. and Reid, D. M. (1971) *Can. J. Bot.* **49**, 967.
[19] Crozier, A. and Reid, D. M. (1972) in *Plant Growth Substances* 1970 (Carr, D. J., ed.), p. 414, Springer, Berlin.
[20] Glenn, J. L., Kuo, C. C., Durley, R. C. and Pharis, R. P. (1972) *Phytochemistry* **11**, 345.

to the countercurrent distribution (CCD) steps shown in Fig. 4. After CCD two fractions *A* (2·15 g) and *BC* (3·4 g) were further purified separately by Sephadex G10[21] and PVP column chromatography.[20] This treatment reduced the weight of fractions *A* and *BC* to 1·03 g and 1·2 g respectively. Neither fraction contained detectable biological activity when tested in the Tan-ginbozu dwarf rice bioassay[22] at 1000- and 10 000-fold dilutions. Extract *A* was chromatographed on a 300 × 40 mm PVP column developed with 0·1 M phosphate buffer (pH 8·0) and 12 consecutive 100 ml fractions were collected. The ethyl acetate-soluble acids were extracted from each fraction and tested for GA-like activity in the dwarf rice bioassay at 500-, 1000-, 2000- and 5000-fold dilutions. Sub-μg levels of GA_3 equivalents were detected in fractions 2–7 which were combined (0·76 g). The extract was then placed on a 140 × 26 mm charcoal–celite 545 (1:2) column which was eluted with 200 ml of 20% aq. acetone, 80 ml of 30% aq. acetone, 280 ml of 80% aq. acetone and 200 ml of 100% acetone. 19 consecutive 40-ml fractions were collected and taken to dryness *in vacuo*. The dwarf rice bioassay indicated the presence of 2·5 μg of GA_3 equivalents in fractions 3–12 which were combined (103 mg).

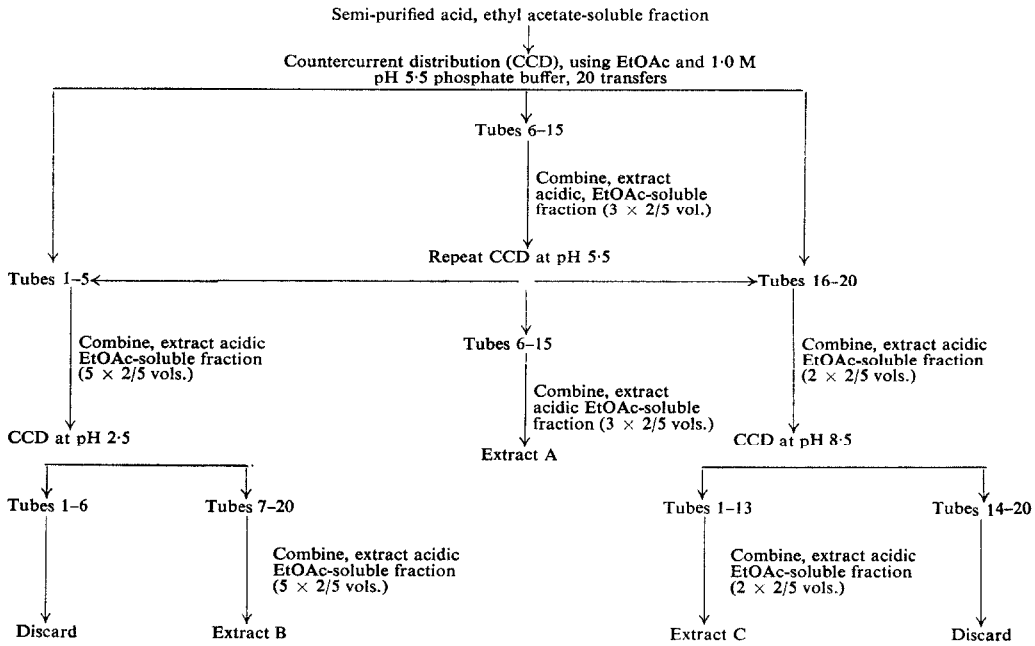

FIG. 4. COUNTERCURRENT DISTRIBUTION OF THE PARTIALLY PURIFIED ETHYL ACETATE-SOLUBLE ACIDS.

Extract *BC* was similarly chromatographed on a PVP column. Biological activity was detected in fractions 2–8 which were combined (0·97 g). Following charcoal–celite column chromatography sub-μg quantities of biological activity were detected in fractions 9–13. These were added to the active fractions from fraction *A* to give a total dry wt of 301 mg.

The extract was next eluted from a 210 × 26 mm charcoal–celite (1:2) column with increasing amounts of acetone in H_2O and 43 fractions were collected. Fraction 1 (250 ml) was eluted in 20% acetone. Fractions 2 and 3 (50 ml each) and fractions 4–39 (25 ml each) were eluted with a gradient from 20 to 100% acetone. Fractions 40–43 (150 ml each) were eluted with 100% acetone. The fractions were taken to dryness *in vacuo* and tested in the dwarf rice bioassay at a 1000-fold dilution. Those fractions exhibiting biological activity were combined and chromatographed on a 26 × 280 mm silicic acid* partition column with a gradient of increasing amounts of ethyl acetate in hexane.[21,23] Successive fractions (80 ml × 25) were collected, taken to dryness *in vacuo* and tested for GA-like activity in the dwarf rice, lettuce hypocotyl and barley

* Mallinckrodt SilicAR CC-4 (Lot JRC).

[21] CROZIER, A., AOKI, H. and PHARIS, R. P. (1969) *J. Exp. Bot.* **20,** 786.
[22] MURAKAMI, Y. (1968) *Bot. Mag. Tokyo* **81,** 33.
[23] POWELL, L. E. and TAUTVYDAS, K. J. (1967) *Nature* **213,** 292.

aleurone bioassays.[22,24–26] The main peaks of biological activity were then individually chromatographed on 70 × 19 mm charcoal–celite (1:2) column eluted stepwise with 100 ml vol. of increasing amounts of acetone in water. The eluates from these columns were taken to dryness, weighed and tested for biological activity in the dwarf rice bioassay. The most active fractions were methylated with ethereal diazomethane and chromatographed using 2% SE33 at 210° on Gaschrome Q (80–100 mesh) packed in a 1·5 m × 6·35 mm glass column, fitted in a Pye 104 gas chromatograph. The flow rate was 75 ml N_2/min. All fractions showed a number of peaks, some of which could be correlated to gibberellins. The methylated fractions were further purified by TLC on silica gel HF developed 2× in 35% v/v, acetone–light petrol. (b.p. 60–80°). The zones of silica gel at the R_fs corresponding to the suspected GAs were eluted with EtOAc and the extracts re-chromatographed on SE33 as before. Further purification prior to GC–MS was found to be necessary. The GA-containing fractions from TLC were therefore purified by preparative GLC using 5% SE33 at 200° on Gaschrome Q columns fitted in a Pye 104 gas chromatograph. The suspected GA peaks were collected from each extract and identification of the GAs was carried out by GC–MS. The methylated and trimethylsilylated methylated fractions from TLC and preparative GLC were chromatographed on both 2% QF1 and 2% SE33 on Gaschrome Q (100–200 mesh) packed in a 1·5 m × 3 mm glass column fitted to an A.E.I. MS 30 mass spectrometer. The helium carrier gas flow of 30 ml/min was removed by a silicone membrane separator. The column temp. was programmed for 200–230° at 2°/min.

[24] BRIAN, P. W., HEMMING, H. G. and LOWE, D. (1964) *Ann. Bot.* **28,** 369.
[25] JONES, R. L. and VARNER, J. E. (1967) *Planta* **72,** 155.
[26] CROZIER, A., KUO, C. C., DURLEY, R. C. and PHARIS, R. P. (1970) *Can. J. Bot.* **48,** 867.