Phytochemistry, 1970, Vol. 9, pp. 2453 to 2459. Pergamon Press. Printed in England.

ENDOGENOUS GIBBERELLINS OF DOUGLAS FIR

A. CROZIER, H. AOKI,* R. P. PHARIS and R. C. DURLEY

Biology Department, The University of Calgary, Calgary 44, Alberta, Canada

(*Received* 6 *February* 1970)

Abstract—Gibberellin A_3 (GA_3) has been characterized by column, TLC and GLC as the major gibberellin in vegetative shoots of coastal (var. *menziesii*) and inland (var. *glauca*) varieties of Douglas fir (*Pseudotsuga menziesii*). Chromatographic data suggest that GA_3 is also the predominant gibberellin in male pollen cones of var. *glauca*. Bioassays of fractions eluted from silicic acid columns showed that vegetative shoots from the coastal and inland varieties contained at least three GA-like compounds in addition to GA_3. The male pollen cones contained at least four additional GAs. The faster-growing coastal variety contained ten times more (GA_3)-like than the more slowly growing inland variety. Shoots of 80-yr-old trees of the inland variety contained five times more GA_3 than tissue from 45-yr-old trees.

INTRODUCTION

GIBBERELLINS have been implicated in growth and differentiation processes of a number of conifer species,[1-9] and gibberellin-like substances have been detected in vegetative shoots of larch (*Larix leptoleptis* L. and *L. decidua* L.),[10,11] Arizona cypress (*Cupressus arizonica* Greene),[12] *Cryptomeria japonica*,[13] Douglas fir (*Pseudotsuga menziesii* var. *menziesii*),[14] berries of juniper (*Juniperus chinensis*),[15] developing embryos of pine (*Pinus jeffreyi* Grev and Balf., *P. ponderosa* Law. and *P. lambertiana* Dougl.)[16] and pollen[17] and vegetative shoots[11] of pine (*P. silvestis* L.). Michniewicz[11] reports that there are seasonal changes in the levels of gibberellin-like substances in pine and larch, and that the period of intensive growth is associated with high GA levels and diminished inhibitor levels. Tree age and relative growth rates were correlated with GA levels.

* Present address: Department of Agricultural Chemistry, University of Nagoya, Nagoya, Japan.

[1] H. HASHIZUME, *J. Japan Forestry Soc.* **41**, 375 (1959).
[2] Y. KATO and N. FUKUHARA, *Abstr. 4th Meeting Jap. Gibberellin Res. Assoc.*, pp. 26 (1960).
[3] Y. KATO, N. FUKUHARA and R. KOBAYASHI, *Abstr. 2nd Meeting Jap. Gibberellin Res. Assoc.*, pp. 67 (1958).
[4] R. P. PHARIS and W. MORF, *Can. J. Botany* **45**, 1519 (1967).
[5] R. P. PHARIS and W. MORF, in *Biochemistry and Physiology of Plant Growth Substances* (edited by F. WIGHTMAN and G. SETTERFIELD), pp. 1341, Runge Press, Ottawa (1968).
[6] R. P. PHARIS, M. D. E. RUDDAT, C. C. PHILLIPS and E. HEFTMANN, *Can. J. Botany*, **43**, 323 (1965).
[7] R. P. PHARIS, M. RUDDAT, C. PHILLIPS and E. HEFTMANN, *Naturwiss.* **52**, 88 (1965).
[8] R. P. PHARIS, M. RUDDAT, C. PHILLIPS and E. HEFTMANN, *Botan. Gaz.* **128**, 105 (1967).
[9] M. RUDDAT and R. P. PHARIS, *Planta* **71**, 222 (1966).
[10] H. HASHIZUME, *Trans. Tottori Soc. Agr. Sci.* **18**, 53 (1965).
[11] M. MICHNIEWICZ, *Wiss. Z. Univ. Rostock, Math. Naturwiss. Reihe.* **4/5**, 577 (1967).
[12] M. RUDDAT, R. P. PHARIS, H. AOKI and A. CROZIER, *Plant Physiol.* **43**, 2049 (1968).
[13] H. HASHIZUME, *Bull. Tottori Univ. Forests.* **4**, 1 (1969).
[14] H. AOKI, A. CROZIER and R. P. PHARIS, Proc. III Symp. on Plant Growth Regulators. In *Zeszyt Naukowe UMK, Biologia* **13** 113 (1969).
[15] J. KATO, W. K. PURVES and B. O. PHINNEY, *Nature* **196**, 686 (1962).
[16] S. L. KRUGMAN, *Forest Sci.* **13**, 29 (1967).
[17] L. MICHALSKI, *Acta Soc. Botan. Pol.* **36**, 475 (1967).

TABLE 1. ORIGIN AND TYPE OF DOUGLAS FIR TISSUES ANALYSED

Tissue	Dry wt.	Age	Origin	Variety
Elongating vegetative shoots	2·0 kg	30 yr	Vancouver Island	*menziesii*
Elongating vegetative shoots	2·4 kg	8–10 yr	Elko, S.E. British Columbia	*glauca*
Elongating vegetative shoots	1·24 kg	45 yr	Kananaskis, W. Alberta	*glauca*
Male pollen cones	2·6 kg	45 and 80 yr	Kananaskis, W. Alberta	*glauca*
Elongating vegetative shoots	0·36 kg	80 yr	Kananaskis, W. Alberta	*glauca*

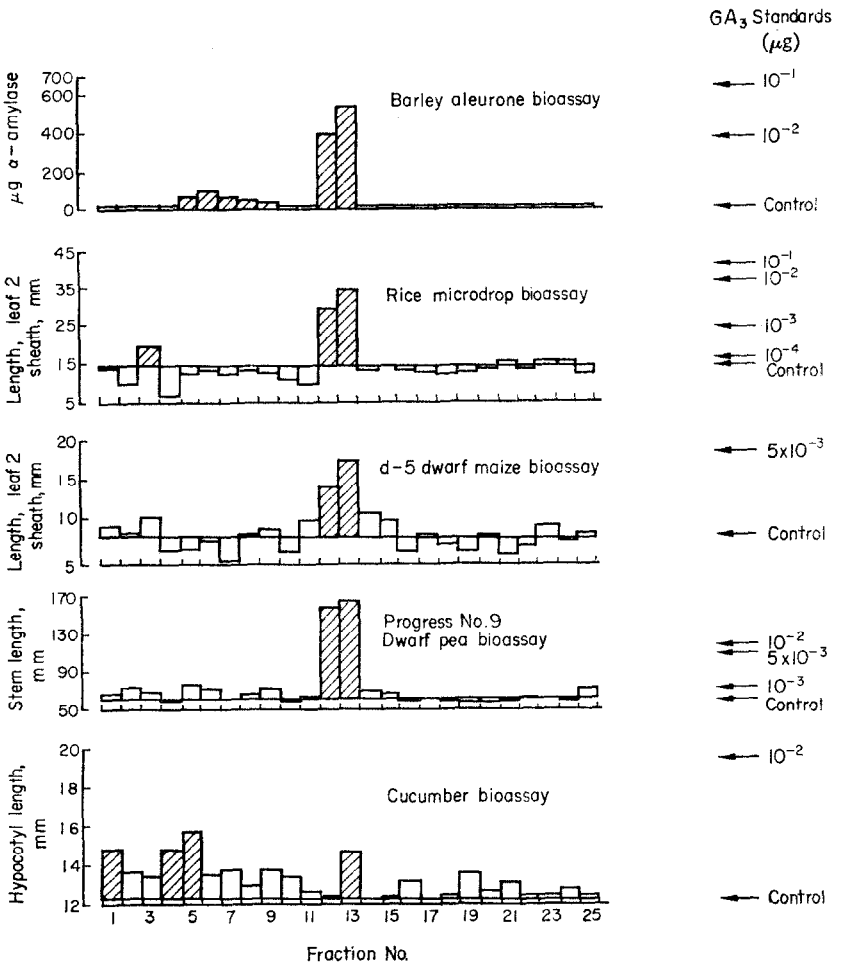

FIG. 1. BIOASSAYS OF A SILICIC ACID PARTITION CHROMATOGRAM OF THE PURIFIED ACIDIC, EtOAc-SOLUBLE FRACTION FROM 2·0 kg OF VEGETATIVE TISSUE FROM 30-yr-old DOUGLAS FIR TREES GROWING ON VANCOUVER ISLAND, BRITISH COLUMBIA.

Dilutions as follows: Barley aleurone bioassay—fractions 5–9 × 20, other fractions × 200; Rice microdrop bioassay—fractions 12 and 13 × 300, other fractions × 100; d-5 Dwarf maize bioassay—fractions 12 and 13 × 150, other fractions × 50; Progress No. 9 Dwarf Pea Bioassay—fractions 12 and 13 × 240, other fractions × 80; Cucumber bioassay—fractions 11–15 × 100, other fractions × 50.

This paper reports the extraction and detection of GA_3 and several other gibberellin-like compounds from elongating vegetative shoots of Douglas fir trees of different ages and from different geographic locations. The endogenous GA's of male Douglas fir pollen cones were also investigated.

RESULTS

Following methanolic extraction of the Douglas fir tissues listed in Table 1, the acidic, ethyl acetate-soluble fraction from each tissue was purified by countercurrent distribution and G-10 Sephadex column chromatography prior to chromatography on a silicic acid partition column. Twenty-five fractions were collected and each was tested for gibberellin-like activity in a number of bioassay systems.

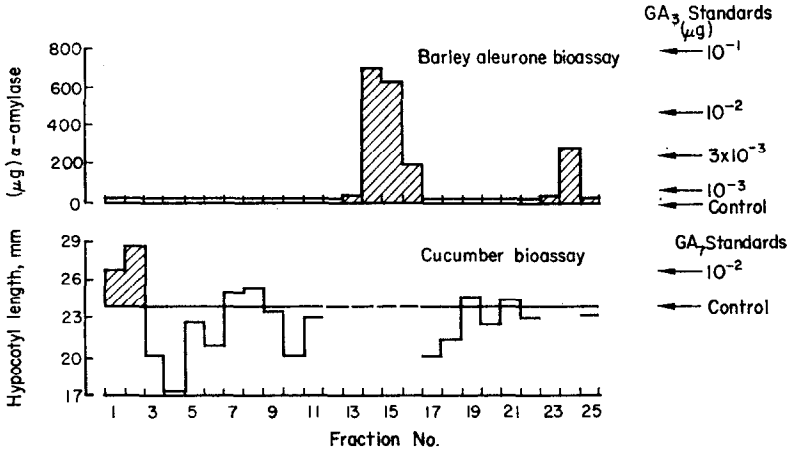

FIG. 2. BIOASSAYS OF A SILICIC ACID PARTITION CHROMATOGRAM OF THE PURIFIED ACIDIC, EtOAc-SOLUBLE FRACTION FROM 2·4 kg OF VEGETATIVE TISSUE FROM 8–10-yr-old DOUGLAS FIR TREES GROWING AT ELKO, S.E. BRITISH COLUMBIA.

Dilutions as follows: Barley aleurone bioassay—all fractions × 22; Cucumber bioassay—fractions 1–11, 17–22 and 25 × 15.

The results of the bioassays are shown in Figs. 1–4. In all extracts the main peak of biological activity was detected between fraction 12 and 15. Gibberellin A_3 is eluted from the silicic acid column in this zone. It was further suggested that this compound was GA_3 by the fact that TLC followed by a barley aleurone bioassay revealed activity at an R_f similar to that of GA_3, and GLC of the trimethyl silyl (TMS) ether on a 2% SE-30 column gave a peak with the same retention time as GA_3. Attempts to obtain a peak corresponding to GA_3 on a 2% QF-1 column were unsuccessful. This was probably due to the small amounts of GA_3 and the partial degradation of the GA_3-TMS ether during its relatively long retention time on the column.

The GA_3 peak in each extract was tested at 100-, 200-, 500-, 1000- and 5000-fold dilutions on the barley aleurone bioassay. The quantitative GA_3/kg estimates for lyophilized tissue are as follows: 30-yr-old Vancouver Island trees–115 μg, 8–10-yr-old trees from S.E. British Columbia—4·8 μg, 45-yr-old vegetative tissue from W. Alberta—1·2 μg, 80-yr-old material from W. Alberta—9·8 μg and 45- and 80-yr-old male pollen cones from W. Alberta—1·65 μg. In addition to variation in GA_3 levels, there were small, but noticeable,

differences in the types of gibberellin-like compounds present in the various tissues (Figs. 1–4).

The tissue from 30-yr-old Vancouver Island trees (Fig. 1) had a band of low activity in fractions 5–9 (barley aleurone), and peaks in fraction 1 (cucumber), fraction 3 (rice) and fractions 4 and 5 (cucumber). The extract of 10-yr-old S.E. British Columbia trees (Fig. 2)

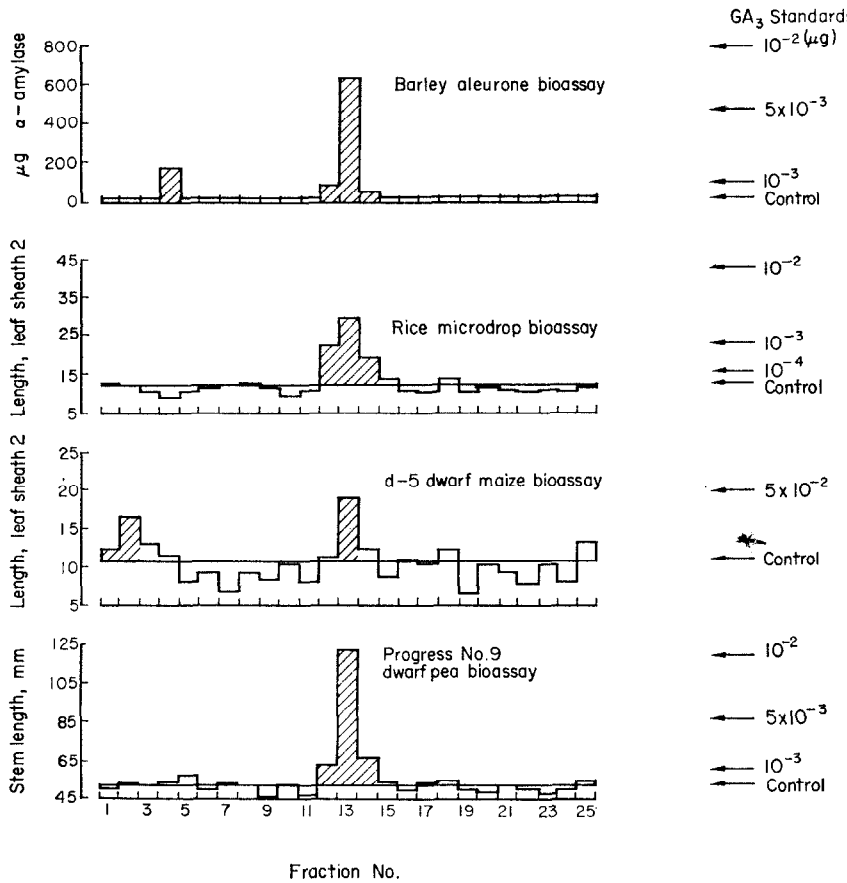

FIG. 3. BIOASSAYS OF A SILICIC ACID PARTITION CHROMATOGRAM OF THE PURIFIED ACIDIC, EtOAc-SOLUBLE FRACTION FROM 1·24 kg OF VEGETATIVE TISSUE FROM 45-yr-old DOUGLAS FIR TREES GROWING AT KANANASKIS, W. ALBERTA.

Dilutions as follows: Barley aleurone bioassay—all fractions × 200; Rice microdrop bioassay—all fractions × 100; d-5 Dwarf maize bioassay—all fractions × 50; Progress No. 9 dwarf pea bioassay—all fractions × 80.

contained activity in fractions 1 and 2 (cucumber), and a peak in fractions 23 and 24 (barley aleurone) that was not detected in any of the other tissues. The vegetative tissue from 45-yr-old W. Alberta trees (Fig. 3) contained peaks in fraction 2 (d-5 maize) and fraction 4 (barley aleurone). The male cones (Fig. 4) showed significant activity in fraction 7 (barley aleurone and rice), fractions 1–5 (rice) fractions 1–4, 6–10 and 20 (pea). The barley aleurone and rice bioassays detected only the GA_3 activity in fractions 13 and 14 in the extract from 80-yr-old vegetative shoots from W. Alberta.

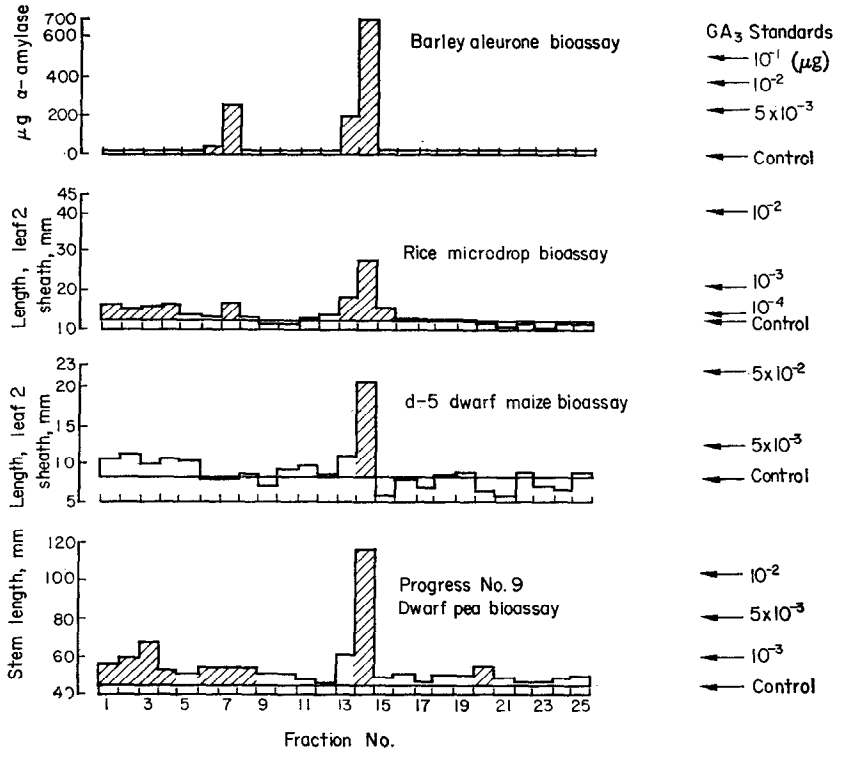

FIG. 4. BIOASSAYS OF A SILICIC ACID PARTITION CHROMATOGRAM OF THE PURIFIED ACIDIC, EtOAc-SOLUBLE FRACTION FROM 2·6 kg OF MALE POLLEN CONES FROM 45 AND 80-yr-old DOUGLAS FIR TREES GROWING AT KANANASKIS, W. ALBERTA.

Dilutions as follows: Barley aleurone bioassay—fractions 5 and 6 × 20, other fractions × 200; Rice microdrop bioassay—all fractions × 100; d-5 Dwarf maize bioassay—all fractions × 50; Progress No. 9 dwarf pea bioassay—all fractions × 80.

DISCUSSION

The vegetative Douglas fir tissues were sampled at essentially the same stage of elongation after bud-break. Ignoring temporarily the age differences of the samples, it is of interest that there is a correlation between relative growth rates[18, 19] and levels of endogenous GA_3. Rapidly growing trees (var. *menziesii*, Vancouver Island) contained an estimated 115 μg of GA_3/kg, the slower growing var. *glauca* from S.E. British Columbia, 4 to 8 μg/kg, and the very slow growing var. *glauca* from W. Alberta contained only 1·2–9·8 μg of GA_3/kg. The only tissues from the same location that differed in age were the 45- and 80-yr-old var. *glauca* samples from W. Alberta. The older tissue contained 9·8 μg of GA_3/kg and the younger shoots only 1·65 μg. This is similar to results obtained by Mirchamiewicz[11] with *Pinus sylvestris*. However, there are a number of variables which should be kept in mind when evaluating these quantitative differences in GA_3 concentrations. The trees from S.E. British Columbia were sexually immature (i.e. juvenile), those from Vancouver Island were just entering

[18] R. E. MCCARDLE, W. H. MEYER and B. DONALD, *U.S. Dept. Agr. Tech. Bull.* **201**, 74 (1949), cited in *U.S. Dept. Agric. Forest. Serv. Agric. Handbook No.* 27 (1965).

[19] R. R. SILEN and L. R. WOIKE, *Proc. N.W. For.* and *Range Expt. Res. Sta. Res. Paper No.* 33 (1959).

sexual maturity, but bore few strobili, while the W. Alberta trees were flowering profusely. It is possible that the presence or absence of a heavy cone crop could affect the concentration of GA's in vegetative shoot tissue. A correlation does exist between heavy cone production and reduced vegetative growth of Douglas fir.[20]

There appear to be qualitative differences in GA's with age, with locality sampled and with tissue differentiation. However, all determinations other than that for GA_3 are by bioassay, and the presence of inhibitory substances may well preclude the detection of low levels of certain GA's.

The main GA in Arizona cypress is GA_3[12, 14] and it is possible to induce precocious "flowering" in this confer with GA_3 treatment.[6] The major gibberellin in both vegetative and reproductive tissue from 45-yr-old trees growing in W. Alberta, also appears to be GA_3 (Figs. 3 and 4). Gibberellin A_3 has been tested on Douglas fir in numerous experiments but no "flowering" response has been observed.[21] It is therefore possible that unlike the situation in Arizona cypress, GA_3 is not intimately involved in strobilus induction in Douglas fir. However, the spectrum of endogenous GA-like compounds in the vegetative and reproductive Douglas fir tissues is somewhat different (Figs. 3 and 4), so perhaps a GA other than GA_3 is effective in the induction process. Alternatively strobilus induction could be brought about by an interaction between GA and other co-factors or hormones. More detailed studies will, however, be necessary before the role(s) of GA in strobilus initiation of Douglas fir can be determined.

EXPERIMENTAL

Tissue

New vegetative shoots from the upper crown of Douglas fir trees were collected during the stage of most rapid elongation and before new buds were visible to the naked eye. The male strobili were collected 5 days before the shedding of pollen. Trees were felled, the tissue was cut and then frozen immediately with liquid N_2 or dry ice. Further information is given in Table 1.

Extraction, Purification and Chromatography

A detailed description of the techniques used are reported by Crozier *et al.*[22] Each batch of tissue was extracted twice with 75% methanol (1 l./100 g tissue). The methanolic extracts were combined, reduced to the aqueous phase *in vacuo* and partitioned against EtOAc at pH 8·0 and then at pH 2·5. The crude acidic extracts were then purified by countercurrent distribution and G-10 Sephadex column chromatography prior to being placed on a silicic acid partition column. The column was eluted with a gradient of increasing concentrations of EtOAc in *n*-hexane.[23] Twenty-five fractions were collected and taken to dryness before being tested for gibberellin-like activity in several bioassays.

Bioassays

Eluates from the silicic acid partition column were tested for gibberellin-like activity in the barley aleurone α-amylase,[24] "Tanginbozu" dwarf rice microdrop,[25] d-5 dwarf maize,[26] Progress No. 9 dwarf pea[27] and cucumber hypocotyl[28] bioassays. The results are plotted as histograms (Figs. 1–4) and the shaded areas indicate activity that is significant at the 5% level.

[20] S. EIS, E. H. GORMAN and L. F. EBELL, *Can. J. Botany* **43**, 1553 (1965).
[21] R. P. PHARIS, unpublished data.
[22] A. CROZIER, H. AOKI and R. P. PHARIS, *J. Exptl. Botany* **20**, 786 (1969).
[23] L. E. POWELL and K. J. TAUTVYDAS, *Nature* **213**, 292 (1967).
[24] R. L. JONES and J. E. VARNER, *Planta* **72**, 155 (1967).
[25] Y. MURAKAMI, *Botan. Mag.* **81**, 100 (1968).
[26] B. O. PHINNEY and C. A. WEST, in *Handbuch Pflanzenphysiol.* **14**, 1184 (1961).
[27] D. KOHLER and A. LANG, *Plant Physiol.* **38**, 555 (1963).
[28] P. W. BRIAN, H. G. HEMMING and D. LOWE, *Anal. Botany London* **28**, 369 (1964).

Thin-layer Chromatography

Portions of the biologically active zones from the silicic acid column were rechromatographed on 0·25 mm layers of Kieselguhr G with CCl_4–HOAc–H_2O (8:3:5). The plates were equilibrated in upper phase for 36 hr, then developed in lower phase plus 20% EtOAc.[29] Developed chromatograms were air dried, zones corresponding to 0·1 R_f units were scraped off the plate and eluted three times with wet EtOAc. The combined eluates were taken to dryness, dissolved in distilled water and tested for gibberellin-like activity with the barley aleurone bioassay. For reference purposes standard gibberellins were run along side extracts and their location determined, after scraping off the chromatographed extracts, by spraying with H_2SO_4/H_2O (7:3) and heating at 120°.

Gas–liquid Chromatography

GLC was carried out on 2% SE-30 and 2% QF-1 columns according to the method of Cavell *et al.*[30]

Acknowledgements—The authors would like to thank Mrs. B. Cosgrove, Mr. J. Glenn and Mr. W. Morf for their technical assistance and Dr. Lorne Doerr, Canada Department of Forestry, Victoria, B.C., Canada, who was instrumental in collecting the Vancouver Island tissue. This work was supported by a Canada Department of Forestry Extramural Research Grant to R. P. Pharis, and a NATO Research Grant to J. MacMillan and R. P. Pharis.

[29] T. KAGAWA, T. FUKINBARA and Y. SUMIKI, *Agri. Biol. Chem.* **27**, 598 (1963).
[30] B. D. CAVELL, J. MACMILLAN, R. J. PRYCE and A. C. SHEPPARD, *Phytochem.* **6**, 867 (1967).