AN INVESTIGATION OF SOME HORMONAL BASES FOR ABSCISSION IN COWPEA (*VIGNA UNGUICULATA* L. WALP.)

BY

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ABSTRACT

The investigations carried out on the abscission problem in cowpea are reviewed. The isolation, characterization, physiological roles, chemistry, biosynthesis and metabolism of the various groups of plant hormones are also reviewed.

Using biological assays and combined gas-liquid chromatography-mass spectrometry (GC-MS), some of the hormones in the extensively purified acidic ethyl acetate extracts obtained from 2-day old and 6-day old cowpea fruits were examined.

Biological assays indicated the presence of only inhibitors in the 2-day old fruits but inhibitors as well as gibberellins and auxins were indicated to be present in the 6-day old fruits.

GC-MS analysis of the extract from 2-day old fruits afforded the identification of the known inhibitors, abscisic acid and phaseic acid. 6'-hydroxymethyl abscisic acid was also identified in the extract and this is the first reported evidence that 6'-hydroxymethyl abscisic acid occurs naturally.

Several plant hormones were identified (GC-MS analysis) in the extract from 6-day old fruits. These were abscisic acid, phaseic acid, dihydrophaseic acid, 'iso'dihydrophaseic acid, 6'-hydroxymethyl abscisic acid; gibberellins A_4, A_6, A_8, 'iso' A_8, A_{17}, and A_{20}.

Gibberellins A_1, A_5 and A_{29} were also believed to be probably present. Two components, believed to be two new gibberellins were also identified in the extract and were tentatively called gibberellins X and Y. Tentative structures were assigned to these two new gibberellins.

Purified acidic ethyl acetate extract obtained from fruits that were over six days old was also analysed on the GC-MS. The result was essentially similar to that obtained for the extract from the 6-day old fruits.

The crude acidic ethyl acetate extracts from 6-day old seeds and the fruit walls of the 6-day old fruits were also examined on the GC-MS. Several gibberellins were tentatively identified in the extract from the seeds but only one gibberellin could be identified in the extract from the fruit walls.

The methyl esters of 16α-hydroxy, 17-hydroxy, and 16α,17-dihydroxy derivatives of gibberellin A_{34} and the 16-epimers of the last two compounds were synthesized from gibberellin A_4. This was done in order to correlate the structures that were tentatively assigned to the two new gibberellins with the natural compounds.

The disparity in the hormonal contents of the 6-day old and 2-day old fruits is discussed in relation to the abscission problem in cowpea.

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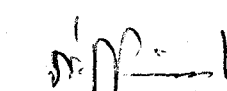

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I N T R O D U C T I O N

Cowpea belongs to the Family Leguminosae of flowering plants. The cowpea is a very important food crop in tropical Africa. Several cultivars of cowpea are cultivated in Nigeria for their dry, edible seeds. These provide a very good source of the much needed protein. They normally grow as spreading, sub-erect or erect annuals with moderately long pods.

Ojehomon[1-4] studied the flowering and fruiting patterns in some cowpea cultivars. The cowpea inflorescence consists of a central peduncle and the flower buds are arranged in four to eight alternate racemes along the peduncle.

Ojehomon[2] reported that, in the cowpea, about 100 to 150 flower buds are produced per plant. Most of these flower buds drop off before they develop into mature fruits. Only about six to sixteen per cent of the flower buds formed ultimately developed into mature fruits. The cowpea therefore exhibits an excessive abscission of buds and immature fruits and this seriously limits the grain yield of the cowpea. It is believed that if abscission can be successfully reduced, the grain yield of cowpea could be increased considerably.

In the cowpea inflorescence, the flowers of the first (lowest) raceme open first followed by those of the second raceme after two to four days[2]. Flower opening thus begins at the bottom of the peduncle and proceeds sequentially upwards. In most cowpea cultivars

like "New Era" and "Mala", only the two lateral flower buds of the first raceme develop into mature fruits. The flowers of the other raceme higher up on the peduncle are shed at different stages of development.

Ojehomon[1,2,4] reported that the flower buds of each raceme however developed into mature fruits when the older fruits below were removed. This implied that the abscission of the buds and immature fruits of the upper racemes was due to the presence of mature fruits on the lowest raceme.

Ojehomon[2] postulated some hypotheses to explain the implication of the lowest, older fruits in the abscission of the upper, younger ones. Two of these hypotheses were: (a) that the older fruits were monopolising the available mobile nutrients and the younger fruits therefore starved and dropped; (b) that the abscission of the flower buds and immature fruits might be stimulated by a substance or substances produced in the older fruits.

Ojehomon[5] tested the first hypothesis by studying the distribution of ^{14}C-assimilates in the cowpea inflorescence using two cultivars of cowpea ("New Era" and "Adzuki"). He administered radioactive carbon-dioxide to the leaves and then used autoradiography to test for the presence of radioactivity in the inflorescence. He found that radioactive assimilates were translocated to all fruits, flowers and flower buds in the inflorescence. This was taken to imply that the

monopolization of nutrients by the older fruits was not an important factor in the abscission of the upper, younger fruits and flower buds.

The occurrence of a monopoly of nutrients by the older fruits was however demonstrated from the results that Adedipe and Ormrod[6] obtained from a study of the distribution pattern of ^{32}P in two cultivars of cowpea. These two cultivars were "Early Ramshorn" which exhibits a relatively high degree of abscission of flowers and fruits; and "Adzuki" which exhibits a relatively low degree of abscission. They showed that there was a preferential accumulation of ^{32}P by the fruits of the lowest raceme in both cultivars. They also showed, quantitatively, that the raceme 1 fruits of "Early Ramshorn", the cultivar that showed a higher abscission degree, were a more potent sink for ^{32}P than those of "Adzuki".

Further studies on the distribution of radioactive nutrients in the cowpea by Adedipe et al[7] also confirmed the above observation. They studied the distribution of ^{14}C in two cultivars of cowpea, "Mala" and "Adzuki". "Mala" exhibits a higher degree of the abscission problem than "Adzuki". They obtained quantitative results that showed that the raceme 1 fruits were more effective in mobilising ^{14}C assimilates away from the younger, upper fruits in "Mala" than in "Adzuki". Their results thus showed that the competition for available mobile nutrients is an important factor in the abscission of flowers and immature fruits.

Extensive investigations[8] by several workers have shown that, generally, in the plant kingdom, abscission is mainly under the control of five plant hormones. These are the anxins, gibberellins, cytokinins, abscisic acid and ethylene. The first three are growth promoters while the last two are growth inhibitors. One important role of the growth promoters in relation to abscission is that of the mobilization of nutrients to developing fruits. Organs which have low concentration of these growth promoters are therefore deprived of nutrients by organs which have higher concentration of the growth promoters.

Seith and Wareing[9] reported that in the French bean (Phaseolus vulgaris), auxin caused mobilization of ^{32}P. They also found that a combination of auxin with gibberellin or auxin with cytokinin was even more effective in the mobilization of ^{32}P.

Adedipe et al[7] also reported that exogenously applied benzyl adenine, a synthetic cytokinin, was effective in redirecting ^{14}C to the advantage of treated fruits in the upper raceme of a variety of cowpea that is known to exhibit a high degree of abscission.

The seed yield of cowpea has been reported to be substantially increased through judicious foliar spray by Aba El-Soad and co-workers[10a]. They sprayed cowpea three times with 25ppm of GA_3 (gibberellic acid) at 10-day intervals. There was an increase in the number of flowers per plant and a reduction in the percentage of

flower drop.

A lot of chemical studies have been carried out leading to the identification and isolation of the various plant hormones. Sensitive chemical methods of analysis have been employed on account of the very low endogenous level of the hormones in plants. These include the various chromatographic and spectroscopic techniques in conjunction with biological assays. These studies have led to the elucidation of the chemical nature of the plant hormones.

The Gibberellins

The gibberellins (GAs) are a group of naturally occurring tetracyclic diterpenoid acids which have a hormonal function in higher plants[10b]. They were initially discovered[10c] as secondary metabolites of the fungus Fusarium moniliforme (Gibberella fujikuroi). This fungus is the causal agent of the bakanae or "foolish seedling" disease of rice. Many higher plants have been shown to contain gibberellins and it is now believed that the gibberellins are present in most, if not all, plants. At present fifty-one[10d] gibberellins are known (Fig.1) of which about forty occur in higher plants. Several glucosyl ethers and esters of gibberellins have also been isolated, and characterized.

1. Physiological roles

The major physiological effects[11,12] include:

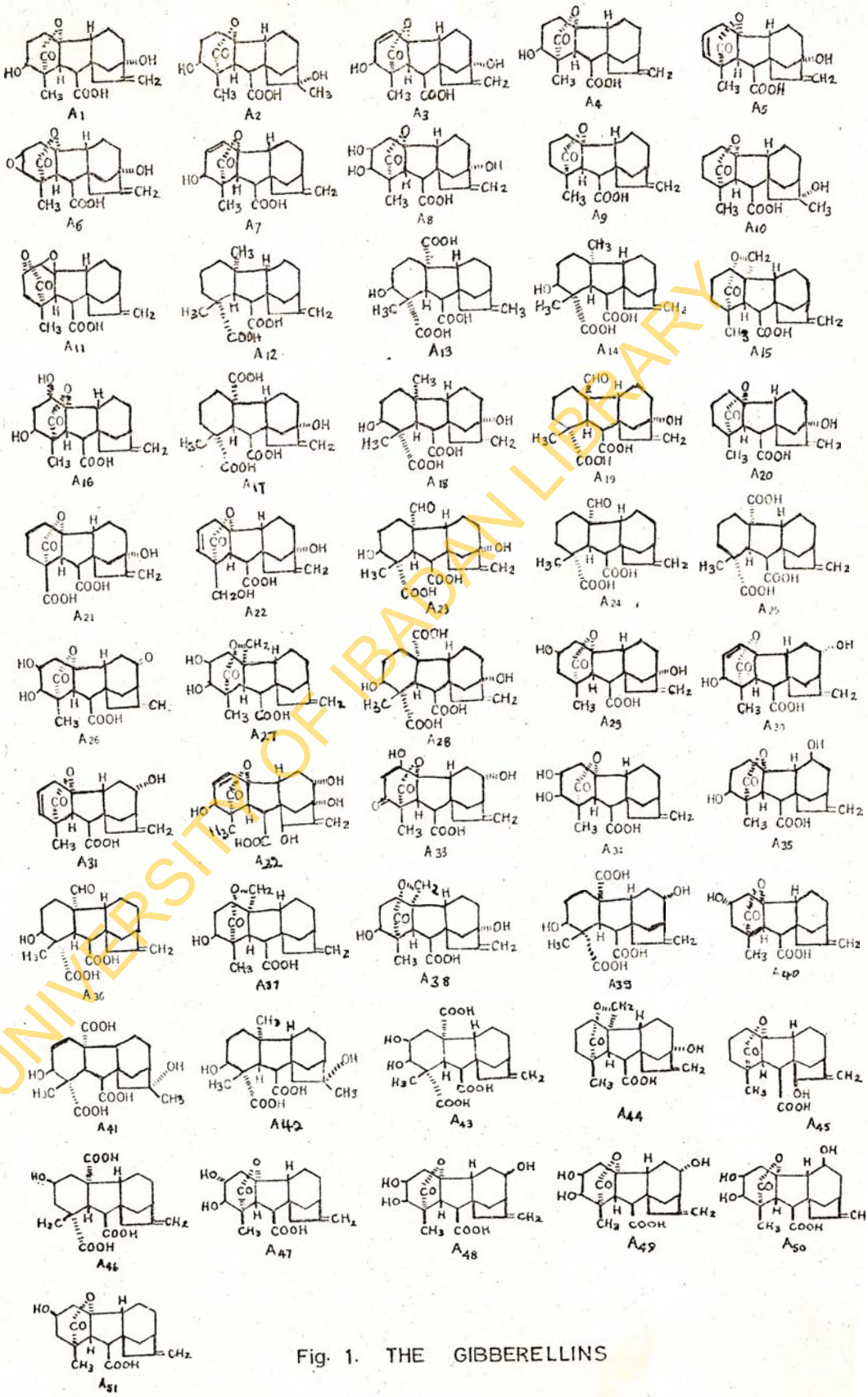


Fig. 1. THE GIBBERELLINS

(a) stimulation of growth extension in many intact plants,

(b) reversal of genetic dwarfism,

(c) induction of stem growth in rosette plants,

(d) stimulation of flowering,

(e) breaking of dormany, and

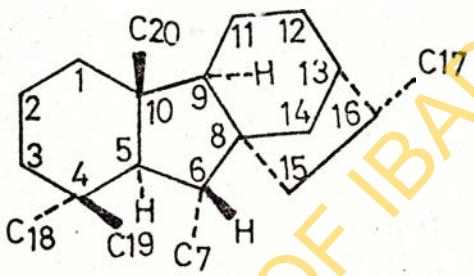
(f) involvement in the synthesis of many enzymes, for example α-amylase in cereal aleurone.

2. Nomenclature

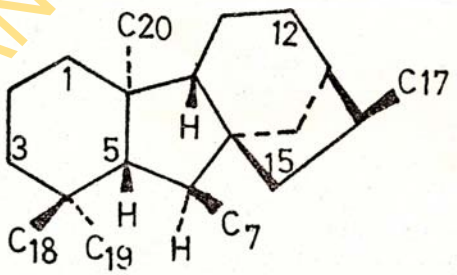
In order to prevent confusion and for mere convenience, the gibberellins have been allocated[13] A-numbers. The known gibberellins have thus been allocated the trivial names GA_1 to GA_{51} (Fig.1).

Apart from the trivial nomenclature used for the gibberellins, there is also a systematic nomenclature[14] based on the I.U.P.A.C. system. This systematic nomenclature is based on the trivial name GIBBERELLANE which is used for the ring system[1] shown in figure 2. The numbering which is as shown is consistent with the numbering of other tetracyclic diterpenes. The gibberellins all have the enantiomeric stereochemistry of the gibberellane skeleton. In the systematic nomenclature, gibberellin A_1 [Fig. 1] is ent - 3α, 10, 13-trihydroxy-20-norgibberell-16-ene-7, 19-dioic acid 19, 10-lactone. The conformational terms α, and β have their normal stereochemical connotations of below and above the plane of the

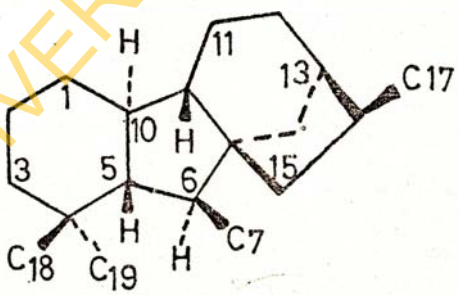
molecule respectively. The connotations are however reversed if the name of the molecule is preceeded by ent. Both the systematic nomenclature and the trivial system are used in this thesis.



[I] GIBBERELLANE



[II] ent-GIBBERELLANE



[III] ent-20-NORGIBBERELLANE

FIG. 2 Nomenclature of GAs.

3. Chemical Structure

The gibberellins can be sub-divided into two groups. These are the C_{20}-gibberellins which have the full complement of diterpenoid carbon atoms and the C_{19}-gibberellins which have lost carbon - 20. The C_{20}-gibberellins are thus derivatives of ent-gibberellane [11] and the C_{19}-gibberellins are derivatives of ent-20-norgibberellane [111].

The C_{19}-gibberellins are characterized by the 19 → 10-lactone ring, with the exception of GA_{11} which has a 19 → 2-lactone. The carbon atoms C-1, C-2, C-3, C-16 and C-17 may be involved in double bond formation while hydroxylation(s) may occur at C-1, C-2, C-3, C-11, C-12, C-13, C-15 and C-16. Carbon-7 is always present as a carboxyl group.

The C_{20}-gibberellins have the C-20 as $-CH_3$, $-CH_2OH$, -CHO, or -COOH groups. Hydroxylations have been found at carbon atoms 2,3 and 13 and carbon atoms 7 and 19 are always present as carboxyl groups. Unlike in the case of C_{19}-gibberellins, double bond occurs only between carbon atoms 16 and 17 in the C_{20}-gibberellins.

4. Structure Determination

The methods employed for the determination of the structures of the gibberellins have become progressively more sophisticated with the development of the various physical methods for the determination of structures of organic molecules. The original

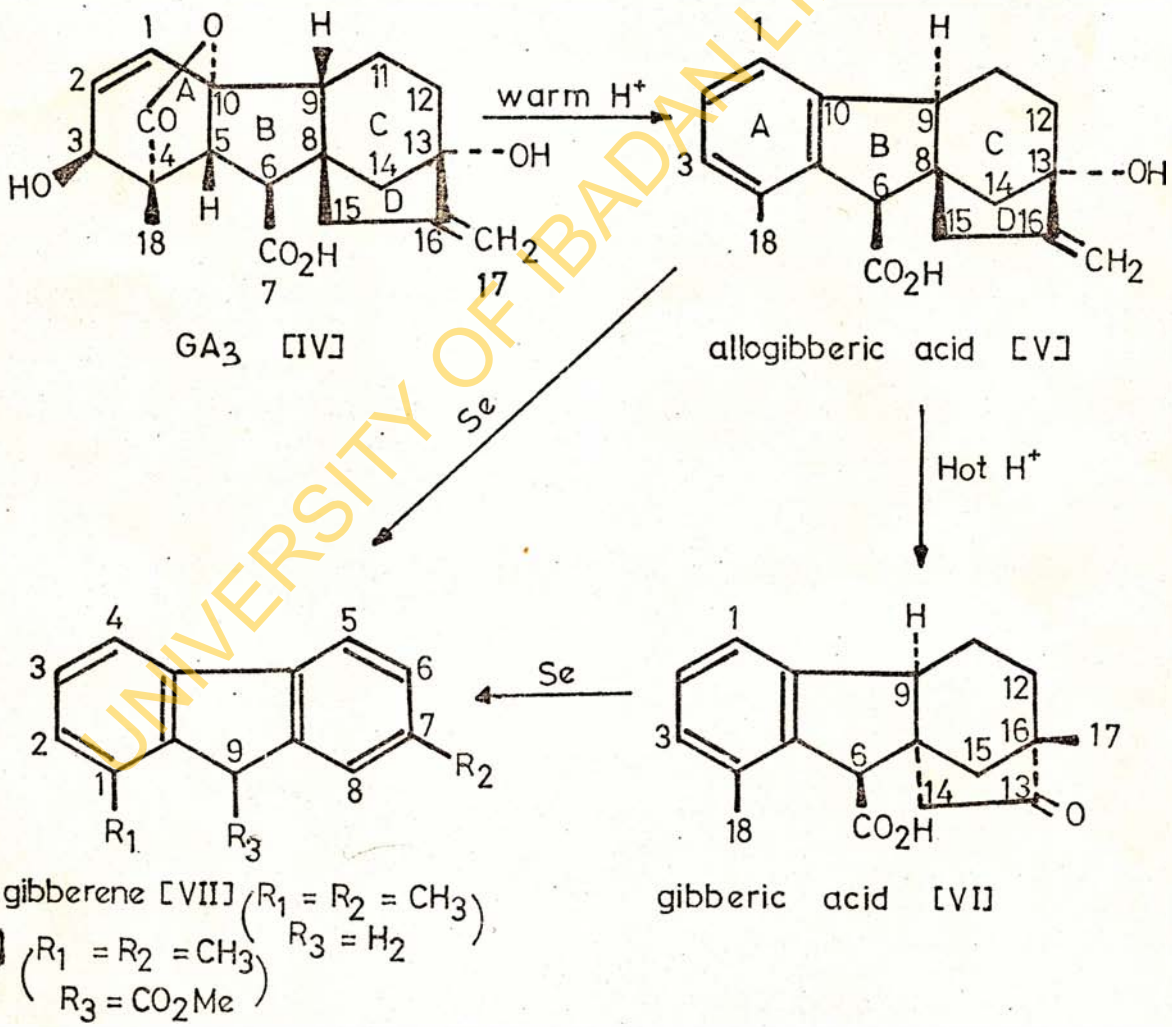
studies on the structural elucidation of the gibberellins centered on GA_3. Gibberellin A_3, also called gibberellic acid is obtained on a large scale from the commercial fermentation of the fungus, Fusarium moniliforme. The structure of GA_3[IV] was determined mainly by chemical methods with the aid of ultraviolet and infrared spectroscopy.

Cross[15] showed that GA_3 was a tetracyclic dihydroxylactonic carboxylic acid. He found that GA_3 formed a monomethyl ester on treatment with excess diazomethane. It also formed a monoacetyl derivative on treatment with acetic anhydride in pyridine. The infra red spectrum of the methyl ester of this monoacetyl derivative of GA_3 still showed a hydroxyl band at 3510cm^{-1}. This showed that there was a second hydroxyl group present and this was considered to be tertiary because of the difficulty of acetylation. The presence of the γ-lactone ring was indicated by the presence of a strong band near 1780 cm^{-1} in the I.R. spectrum of GA_3. Hydrognation showed the presence of two carbon, carbon double bonds.

Further information on the structure of GA_3 was obtained from the elucidation of the structures of some degradation products of GA_3. These were allogibberic acid[V], gibberic acid[VI], and gibberene[VII]. Allogibberic acid was obtained[15] when GA_3 was treated with dilute HCl at 55-65° for about $2\frac{1}{4}$ hours. Gibberic acid was obtained as the main product when either GA_3 or allogibberic

acid was refluxed with dilute hydrochloric acid for about 1 hour. Gibberic acid and allogibberic acid both gave gibberene on dehydrogenation with selenium.

SCHEME 1



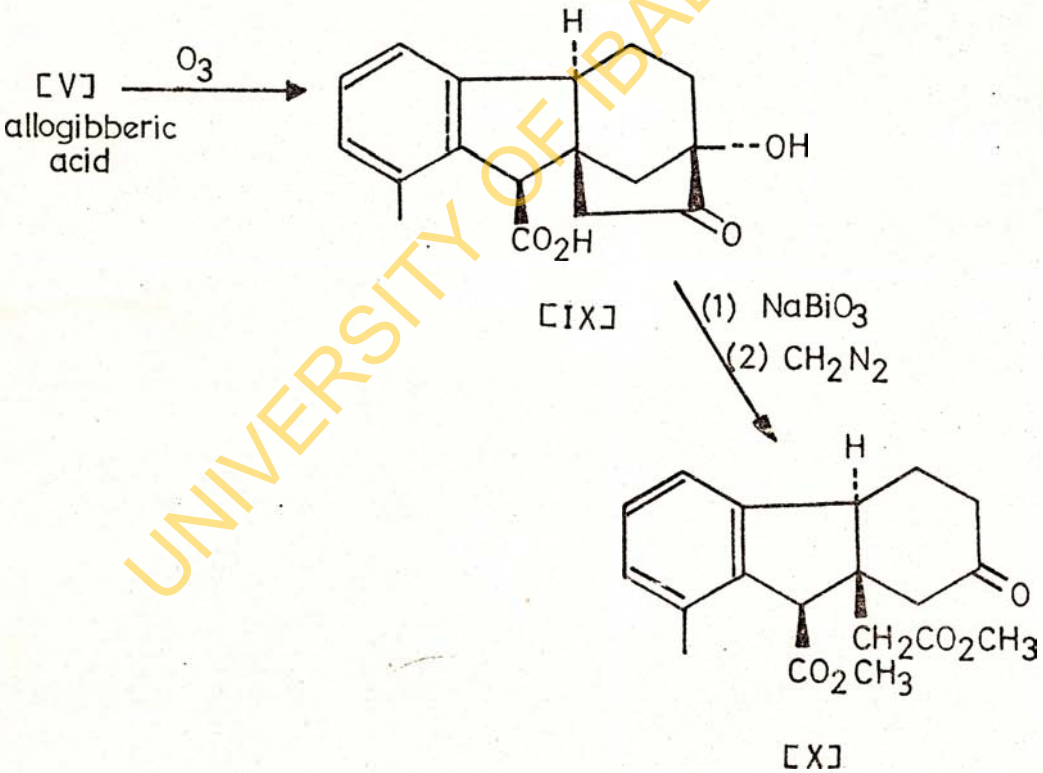
Mulholland and Ward[16] showed that gibberene was 1,7-dimethyl-fluorene[VII] by oxidative degradation to the known fluorene-1, 7-dicarboxylic acid. This structure for gibberene was confirmed by synthesis[16].

Gibberic acid was deduced to be a keto-acid because it formed an ester and an oxime. The I.R. spectrum showed a band at 1741 cm^{-1} indicating that the ketone group was present in a five-membered ring[15]. The presence of the hexahydrofluorene nucleus was established[17] by selenium dehydrogenation to 1,7-dimethylfluorene, (gibberene)[VII]. The position of the carboxyl group was determined[17] by the degradation of gibberic acid to methyl 1,7-dimethyl-fluorene-9-carboxylate[VIII]. This was identical to a specimen prepared by carboxylation of the 9-lithium derivative of 1,7-dimethylfluorene with solid carbon dioxide, followed by methylation with diazomethane. The structure of gibberic acid was finally established[17] to be as shown in structure[VI].

The structure of allogibberic acid [V] was determined as follows. Hydrogenation showed the presence of an ethylenic double bond which was shown to be exocyclic because ozonolysis of allogibberic acid gave formaldehyde and a nor-ketone[IX][18]. The methyl ester of allogibberic acid was isomerised with acid to methyl gibberate. This showed that the carboxyl group was at the same position as in gibberic acid. The I.R. spectrum of methyl allogibberate showed an absorption at 3460 cm^{-1} indicating the

presence of a hydroxyl group. This hydroxyl group was deduced to be tertiary because of the difficulty of acylation. The structure of allogibberic acid was established as [V] and the absolute configuration followed from measurements of optical rotatory dispersion on the keto-ester[X][19,20] obtained from the nor-ketone[IX] by oxidation with sodium bismuthate followed by methylation with diazomethane.

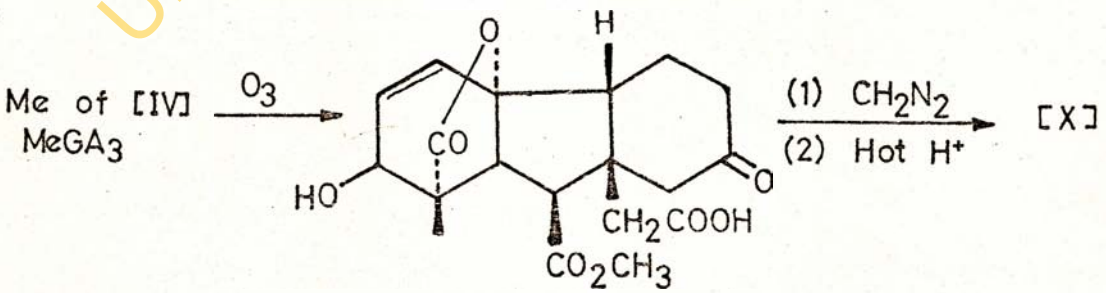
SCHEME 2



[X]

The elucidation of the structure of allogibberic acid paved way for more progress in the determination of the structure of gibberellic acid (GA_3). As stated earlier, Cross[15] had shown that GA_3 was a tetracyclic dihydroxy lactonic carboxylic acid with two carbon, carbon double bonds. The methyl esters of both gibberellic acid and allogibberic acid gave methyl gibberate on refluxing with dilute HCl. This indicated that the position of the carboxyl group in both GA_3 and allogibberic acid were the same. The ozonolysis of methyl ester of GA_3 gave formaldehyde and ultimately a keto-acid[XI].[21] The methyl ester of this keto-acid gave the keto-ester[X] on treatment with acid. This same keto-ester had also been obtained by oxidation, followed by methylation of the nor-ketone[IX] obtained from the ozonolysis of allogibberic acid. This showed[21] that the B/C/D rings of both GA_3 and allogibberic acid were

SCHEME 3



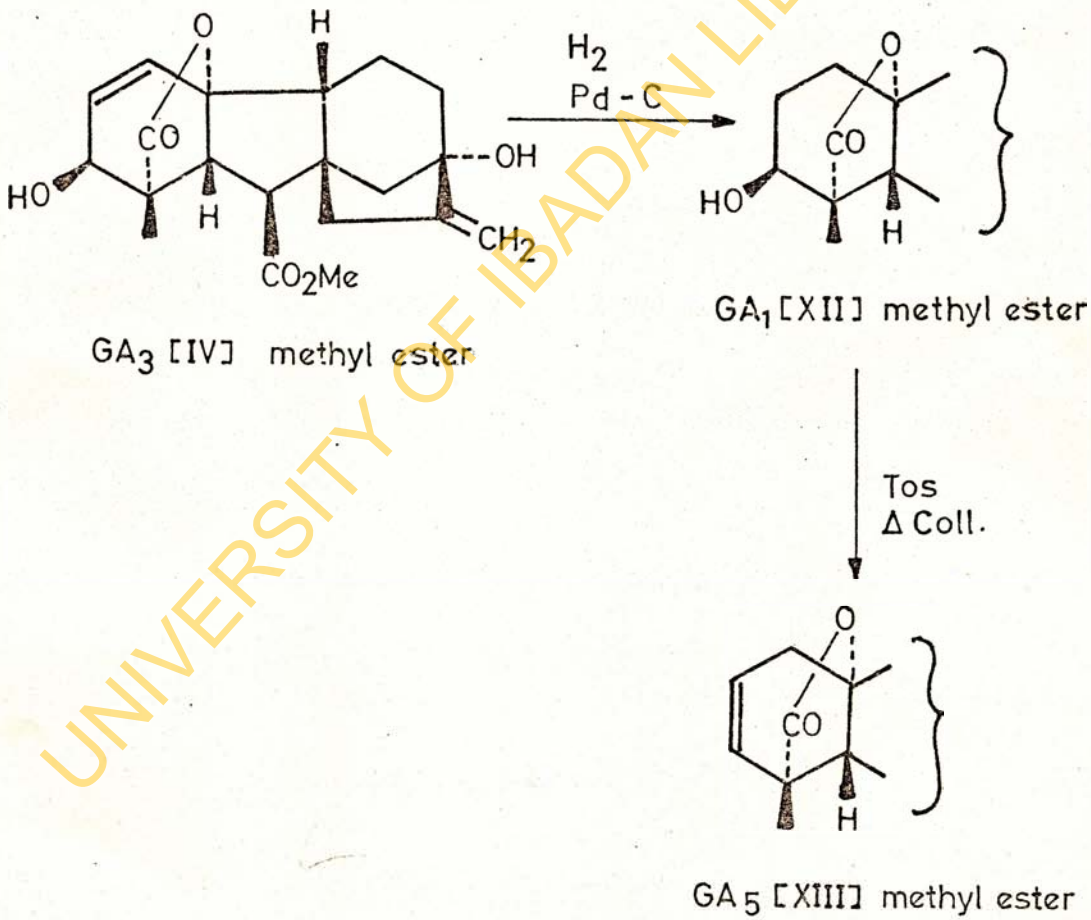
[XI]

similar, and the formation of allogibberic acid from GA_3 therefore involved only the aromatisation of ring A. The γ-lactone and the secondary hydroxyl group must therefore be in ring A of GA_3. The hydroxyl group was shown to be allylic by oxidation with manganese dioxide to give an α,β-unsaturated ketone. The ring A of GA_3 must also contain the methyl group which appeared in position 1 of the fluorene degradation products. There was a high yield of acidic hydrogenolysis products on catalytic hydrogenation of the ring A double bond of the methyl ester of GA_3. This was taken to imply an allylic lactone system. The position of the γ-lactone was deduced from this. The position of the allylic hydroxyl group was established[21] by stepwise degradation. The correct structure of GA_3 was finally deduced by Cross et al[22] and the deduction was supported by nuclear magnetic resonance studies[23]. There were some controversies about the orientation of the lactone ring[24] and the 9 - H was considered to have an α-configuration. These controversies were finally resolved through x-ray and circular dichroism studies[25-27]. The full structure and stereochemistry of GA_3 was thus established as shown in [IV].

The structure of many of the other C_{19}-gibberellins were established by relating them chemically to GA_3 either directly or indirectly. For example GA_1 [XII] was shown to be dihydroGA_3 by the

fact that controlled hydrogenation of $MeGA_3$ with palladium-carbon catalyst (stopping after the absorption of 0.94 mole of H_2) gave $MeGA_1$[28].

SCHEME 4



GA_3 [IV] methyl ester

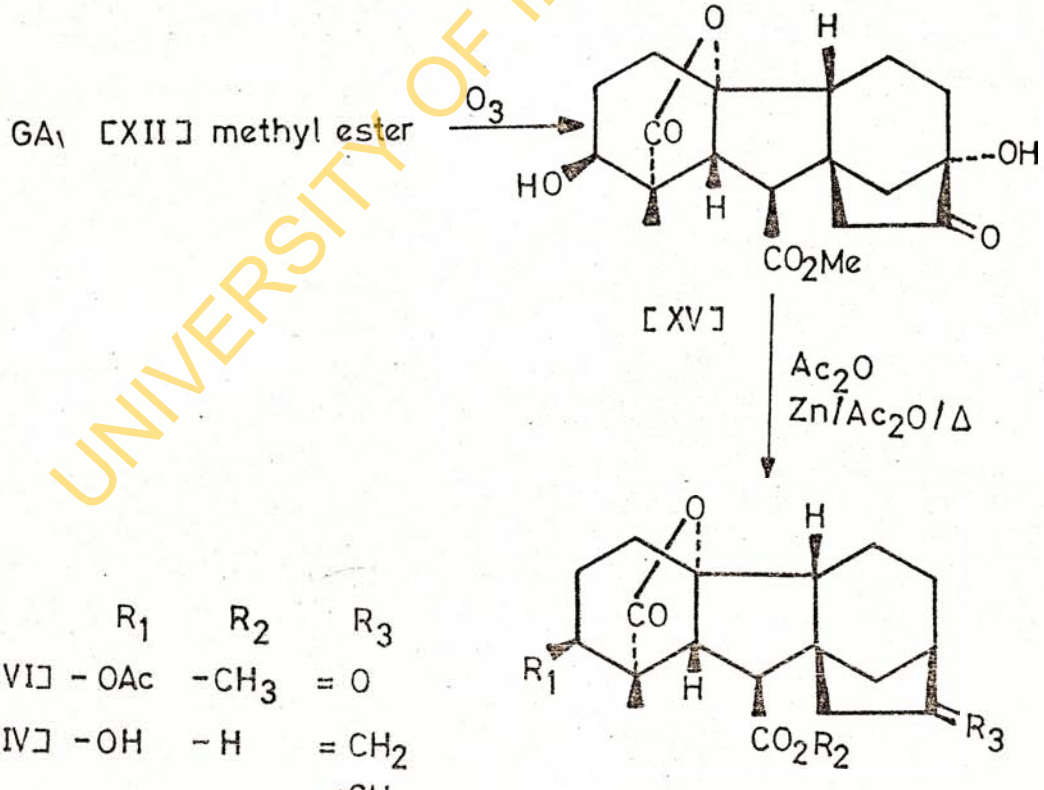
GA_1 [XII] methyl ester

GA_5 [XIII] methyl ester

Dehydration of $MeGA_1$, by refluxing the 3-toluene-p-sulphonyl derivative of $MeGA_1$ in collidine gave GA_5 [XIII] as the methyl ester[29]. Thus GA_5 was established as dehydrated GA_1.

GA_4 [XIV] was related to GA_1 as follows: Ozonolysis of $MeGA_1$ gave the nor-ketone[XV] Acetylation of this nor-ketone followed by reductive de-acetoxylation (by boiling for 36 hours with zinc and acetic anhydride) of the resulting diacetate gave the mono acetate of MeA_4 nor-ketone [XVI][30].

SCHEME 5



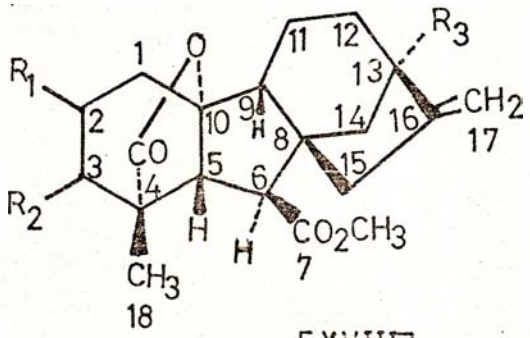
	R_1	R_2	R_3
[XVI]	-OAc	$-CH_3$	= O
[XIV]	-OH	-H	$= CH_2$
[XVII]	-OH	-H	$\begin{matrix} CH_3 \\ OH \end{matrix}$

GA_2 [XVII] was established as hydrated GA_4 [XIV][31]. This was because treatment of GA_4 with dilute hydrochloric acid for two days at room temperature gave GA_2.

Since the time after the determination of the structure of GA_3, there have been a lot of progress in the development of physical methods for the determination of the structures of organic compounds. Nuclear magnetic resonance spectroscopy and mass spectrometry in particular have been extensively used in the determination of the structure of the numerous other gibberellins.

(a) NMR Spectroscopy

Hanson[32] reported the nuclear magnetic resonance of some gibberellin derivatives in deuterochloroform ($CDCl_3$) and deuteropyridine (C_5D_5N). Since then there have been reports of the NMR of the various other gibberellins that have been isolated, and the data of the chemical shift of some gibberellins have been published[32-35] [Table 1].



[XVIII]

The 5-H resonance and the 6-H resonance are very important characteristic features of the proton nuclear magnetic resonance spectra of gibberellins. The 5-H is a β-axial substituent on ring A and therefore 1,3-diaxial transannular effects operate from the 3 position. The position of the 5-H resonance is generally very susceptible to substitution in ring A. Thus in gibberellin A_9 methyl ester (XVIII], $R_1 = R_2 = R_3 = H$) the 5-H resonance appears at δ2.47 in $CDCl_3$. In gibberellin A_4 methyl ester ([XVIII], $R_1 = R_3 = H$, $R_2 = \beta OH$), there is a downfield shift of the 5-H resonance to δ3.15. This deshielding is consistent with transannular 1,3-diaxial interaction. In the 3-epimer of GA_4 methyl ester (3-αOH), the position of the 5-H resonance is very near that observed for GA_9 methyl ester since the 1,3-diaxial interaction is absent in the structure of the 3-epimer of gibberellin A_4 methyl ester. The deshielding due to 1,3-diaxial interaction is amplified in deuteropyridine. This effect is therefore very useful in determining the stereochemistry of the substituent on C-3. Unlike the 5-H, the 6-H is an α-substituent on ring B. The chemical shift for the 6-H is usually fairly constant within the range δ2.83 for most C_{19}-gibberellin methyl esters in deuterochloroform.

The C-17 methylene protons usually give two broad peaks at around δ5.0 in deuterochloroform. The presence of a 13-hydroxyl group causes a significant downfield shift in the position of one of these

peaks. This downfield shift is further increased when the NMR spectrum is determined in deuteropyridine. A comparison of the chemical shifts of the C-17 protons in gibberellin A_9 methyl ester (XVIII, $R_1 = R_2 = R_3 = H$) and gibberellin A_1 methyl ester (XVIII, $R_1 = H$, $R_2 = R_3 = OH$) in both deutrochloroform and deuteropyridine in Table 1a will illustrate this point. This phenomenon is therefore useful in determining whether or not the 13-hydroxyl is present.

The chemical shift of the C-18 methyl protons is sensitive to the presence of other substituents in ring A.

Table 1a

Chemical Shift (δ,ppm) of some protons in some C_{19}-gibberellin methyl esters, after Takahashi, N[33].

	C_4-CH_3	C_{16} = CH_2	H - 1	H - 2	H - 3	H - 5	H - 6
A_1 (i)	1.13	5.23, 4.92			3.75	3.17	2.65
(ii)	1.44	5.59, 5.04			4.08	3.72	2.97
A_2 (ii)	1.45	1.45 (CH_3)			4.08	3.79	3.02
A_3 (i)	1.23	5.25, 4.94	6.30	5.87	4.08	3.17	2.75
(ii)	1.54	5.45, 5.02	6.41	6.11	4.49	3.69	3.05
A_4 (i)	1.13	4.95, 4.83			3.80	3.15	2.65
(ii)	1.43	4.96, 4.86			4.06	3.68	2.93
A_5 (i)	1.22	5.21, 4.92		5.80	5.65	2.60	2.77
(ii)	1.33	5.60, 5.06		5.69	5.69	2.88	2.99
A_7 (i)	1.23	4.95, 4.83	6.32	5.87	4.12	3.25	2.73
(ii)	1.54	4.97, 4.85	6.37	6.04	4.42	3.62	2.97
A_8 (ii)	1.71	5.61, 5.03		4.38	4.22	4.01	3.17
A_9 (i)	1.07	4.91, 4.79				2.47	2.68
(ii)	1.12	4.97, 4.87				2.61	2.86
A_{10} (i)	1.1	1.38 (CH_3)				2.47	2.77
A_{16} (i)	1.12	4.95	4.20		3.95	3.20	2.72
A_{20} (i)	1.07	5.21, 4.90				2.50	2.67
(ii)	1.15	5.58, 5.05				2.70	2.91

Table 1a (contd.)

	C_4-CH_3	C_{16} = CH_2	H - 1	H - 2	H - 3	H - 5	H - 6
A_{21}(i)		5.20, 4.90				3.13	2.76
(ii)		5.58, 5.06				3.42	3.05
A_{22}(i)	3.75(CH_2OH)	5.21, 4.90				2.83	2.83
A_{26} (i)	1.21	5.20, 5.05		3.87	3.75	3.37	2.73
(ii)	1.50	5.25, 5.00		4.30	4.15	3.91	3.03
A_{29} (ii)	1.26	5.06, 5.05		4.40		2.95	3.10
A_{32} (ii)	1.68	5.77, 5.72	6.48	5.95	4.50	4.05	3.37
A_{34} (ii)	1.71	5.00, 4.87		4.40	4.23	3.98	3.16
A_{35} (i)	1.15	5.04, 4.91			3.86	3.26	2.74
A_{40} (i)	1.10	4.96, 4.84		4.28		2.74	2.58
(ii)	1.27	5.00, 4.90		4.49		3.00	2.82

(i) NMR determined in $CDCl_3$

(ii) NMR determined in C_5D_5N

Table 1b

Chemical Shifts (δ,ppm) of protons in some C_{20}-gibberellin methyl esters, after Takahashi N[33].

	C_4-CH_3	C_{10} substituents	C_{16} = CH_2	H -2	H - 3	H - 5	H - 6
A_{13} (i)	1.21		4.80, 4.73		3.89	2.51	3.79
A_{15} (i)	1.15	(CH_2OCO) 4.03, 4.42	4.90, 4.75			2.21	2.79
A_{17} (i)	1.09		5.06, 4.83				3.72
(ii)	1.23		5.42, 4.95				4.05
A_{19} (i)	1.15	9.73(CHO)	5.02, 4.80			2.43	3.87
(ii)	1.28		5.57, 5.10			2.46	4.16
A_{23} (i)	1.20	9.72(CHO)	5.17, 4.93		4.11	2.77	3.88
(ii)	1.60		5.53, 5.03			3.37	
A_{24} (i)	1.11	9.62(CHO)	4.84, 4.76			2.19	3.81
A_{25} (i)	1.10	(CH_2OCO)	4.80, 4.73			2.07	3.78
A_{27} (i)	1.24	4.46, 4.42	4.95, 4.83	3.86	3.72	2.76	2.76
(ii)	1.69	4.58, 4.19	4.93, 4.79	4.35	4.18	3.27	3.04
A_{28} (i)	1.22		5.14, 4.91		3.99	2.58	3.78
(ii)	1.67		5.53, 5.05		4.40	3.22	4.28
A_{36} (i)	1.22	9.68 (CHO)	4.92, 4.84		4.11	2.75	3.91
(ii)	1.59	9.96 (CH_2OCO)	4.94, 4.86		4.48	3.34	4.29
A_{37} (i)	1.19	4.42, 4.07	4.90, 4.78		3.74	2.74	2.74

(i) $CDCl_3$ (ii) C_5D_5N

The resonance usually appearing as singlet is deshielded by the presence of either hydroxyl group or double bond in ring A. (Tables 1a & 1b). As an illustration, in $CDCl_3$, the chemical shift of the C-18 methyl protons of GA_9 methyl ester (XVIII, $R_1=R_1=R_3=H$) occurs at δ1.07 whereas it occurs at δ1.13 in GA_1 methyl ester (XVIII, $R_1=H$, $R_2=\beta OH$, $R_3=\alpha OH$). The downfield shift is even more prominent in the NMR spectrum of gibberellin A_3 methyl ester (δ1.23) which apart from having the 3-hydroxyl also has a 1-2 double bond. The deshielding of the C-18 methyl protons caused by the presence of a ring A double bond can be distinguished from that caused by a hydroxyl substituent in ring A by determining the NMR in $CDCl_3$ and deuteropyridine. Whereas deuteropyridine increases the deshielding due to hydroxyl substituent it does not affect the deshielding due to the double bond.

(b) Mass Spectrometry

Mass spectrometry is another technique that has become extremely useful in the determination of the structures of the gibberellins. There have been reports[36-39] of the high and low resolution mass spectra of gibberellin methyl esters and the low resolution mass spectra of the trimethylsilyl ethers of the methyl esters of many of them have been published[39].

(i) Methyl Esters

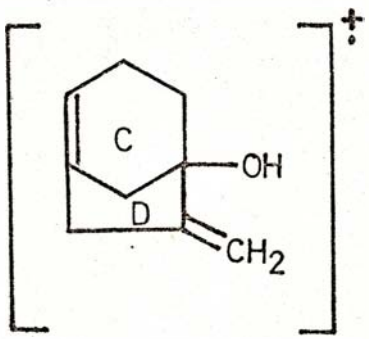
Most C_{19}-gibberellin methyl esters show the molecular ion with moderate intensity. They also show prominent peaks at M^+-32, M^+-44, M^+-46, M^+-60, M^+-62, M^+-104, M^+-106, and M^+-122[38]. The M^+-32 and the M^+-60 peaks are due to the loss of CH_3OH and CH_3COOH respectively from the methoxycarbonyl group attached to C-6. The M^+-44 and the M^+-46 peaks are associated with the loss of CO_2 and HCOOH respectively from the lactone in ring A. The M^+-62 peak is a characteristic feature of the mass spectra of GAs that have hydroxyl group in ring A. The peak which is normally prominent is due to the concerted loss of CO_2 and H_2O from the lactone and the hydroxyl group respectively. The M^+-62 ion is absent in the mass spectra of gibberellins that lack hydroxyl group in ring A. The prominent peaks at M^+-104 and M^+-106 are due to the loss of $HCOOCH_3 + CO_2$ and $HCOOH + HCOOCH_3$ respectively. They are present in the mass spectra of the methyl esters of most C_{19}-gibberellins. The M^+-122 ion is due to the loss of $HCOOCH_3 + H_2O + CO_2$. The M^+-104, M^+-106 and M^+-122 ions thus indicate that the loss of CO_2 or HCOOH from the lactone in ring A and the elimination of the methoxycarbonyl group attached to C-6 can occur concurrently.

The mass spectra of the methyl esters of the C_{20}-gibberellins also show the molecular ion with moderate intensity and prominent peaks at M^+-32 and M^+-60, like those of the C_{19}-gibberellins. The M^+-44, M^+-46 and the M^+-62 ions which are associated with the elimination

of the lactone in ring A are not normally present. Another characteristic feature of the mass spectra of the methyl esters of the C_{20}-gibberellins is the presence of a prominent peak at M^+-119/120 which is due to the loss of two moles of $HCOOCH_3$ from two methoxycarbonyl groups. This can be used to distinguish the C_{20}-gibberellins from the C_{19}-GAs since the C_{19}-gibberellins are monocarboxylic and therefore cannot give rise to the M^+-119/120 ion. The only exception is GA_{21} which is the only C_{19}-gibberellin known that is dicarboxylic. The mass spectra of the methyl esters of the C_{20}-gibberellins also contain peaks at M^+-91/92 which are believed to arise from the loss of 59/60 ($HCOOCH_3$) from one methoxycarbonyl group and the loss of 31/32 (CH_3OH) from a second methoxycarbonyl group.

M^+-18 ions are present in the mass spectra of the methyl esters of the hydroxylated gibberellins. They are also present in the mass spectra of methyl esters of GA_6 and GA_{11} which have epoxides in ring A.

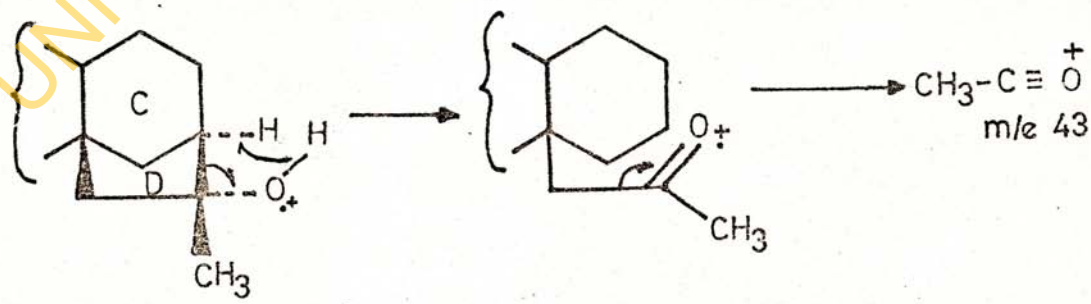
The mass spectra of the methyl esters of gibberellins that have C-13 hydroxyl, are characterised by intense peak at m/e 136. This is believed[38] to be fragment[XIX] formed from rings C and D.



[XIX]

Gibberellins that have the C-16 hydroxyl, for example GA_2 and GA_{10} have intense base peaks at m/e 43 [XX] in the mass spectra of their methyl esters. This ion is believed[39] to arise from ring D by the fragmentation pattern [XXI] → [XX].

SCHEME 6



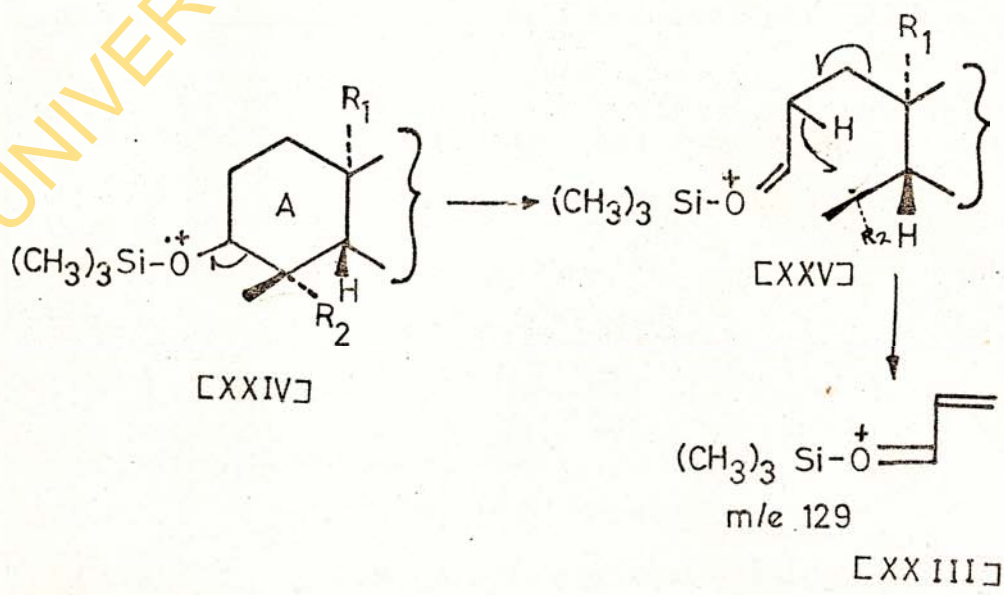
[XXI] [XXII] [XX]

(ii) Trimethylsilyl ethers

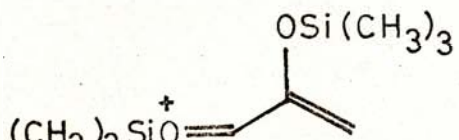
The mass spectra of the trimethylsilyl ethers of the gibberellin methyl esters are very diagnostic of the structures of the hydroxylated gibberellins. The low resolution mass spectra of the trimethylsilyl ethers of some gibberellins have been published by Binks et al[39]. The mass spectra contain M^{+}-31/32 and M^{+}-59/60 ions associated with the methoxycarbonyl group just as in the mass spectra of the methyl esters. Peaks at M^{+}-15 ($-CH_3$), M^{+}-89/90 [$(CH_3)_3SiO-$], m/e 73 and m/e 75 [$(CH_3)_3Si-$] are associated with the trimethylsilyl ether group.

Hydroxylation at C-3 is characterized by the presence of prominent ion at m/e 129 which is accompanied in some cases by an M^{+}-129 peak. The ion at m/e 129 is believed[39] to have the structure [XXIII], arising from ring A by the process shown in Scheme 7 below.

SCHEME 7

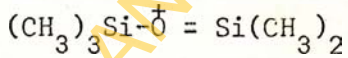


The mass spectra of gibberellins having the 2,3-diol are characterized by the presence of an ion at m/e 217 corresponding to [XXVI], arising from ring A. They also show an ion [XXVII] at m/e 147 which is believed to come from the rearrangement of the vicinal, 2,3-trimethylsilyl ether groups.



m/e 217

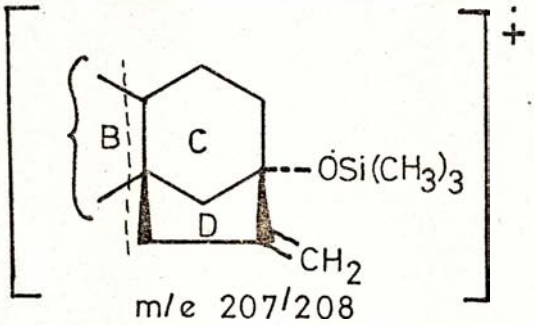
[XXVI]



m/e 147

[XXVII]

The mass spectra of gibberellins that have hydroxyl attached to C-13 have intense molecular ions which are usually the base peaks. The molecular ion is of much lower intensity in the mass spectra of gibberellins that lack the 13-hydroxyl group. The mass spectra of the 13-hydroxylated gibberellins are also characterized by prominent peaks at m/e 207/208 [XXVIII] formed from rings C and D.

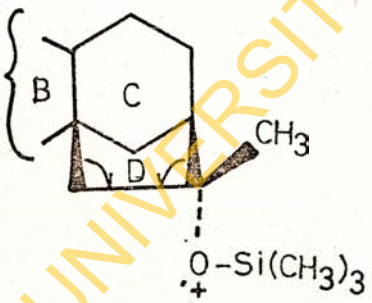


m/e 207/208

[XXVIII]

16-hydroxylated gibberellins are characterized by intense peak which is the base peak at m/e 130. This ion [XXIX] is derived from cleavage of ring D probably by the process [XXX] → [XXIX]

SCHEME 8



[XXX]

→ $CH_2 = \overset{CH_3}{\underset{|}{C}} - \overset{+}{O} - Si(CH_3)_3$

m/e 130

[XXIX]

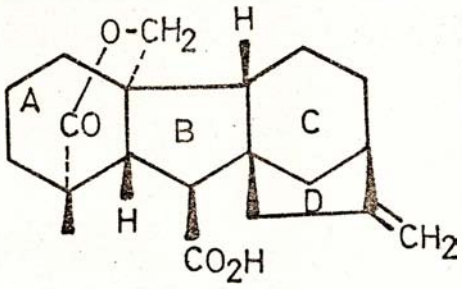
The trimethylsilyl ethers of the C_{20}-gibberellins show prominent $M^{+}-90$, $M^{+}-91$, or $M^{+}-92$ ions just like the methyl esters. These are due to the loss of 59/60 ($-HCOOCH_3$) from one methoxycarbonyl group and loss of 31/32 ($-CH_3OH$) from a second methoxycarbonyl group. The mass spectra of the C_{20}-gibberellins that have the C-20 as aldehyde group (GA_{19}, GA_{23}, GA_{36}) have intense base peaks at $M^{+}-28$ corresponding to the loss of CO from the aldehyde function.

5. Synthesis of the gibberellins

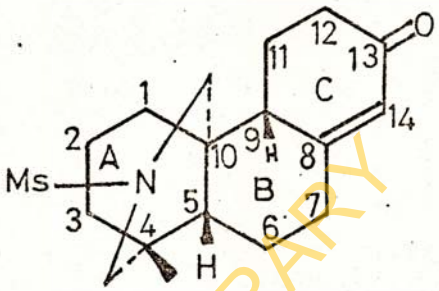
There have been reports[40,41] in literature of the stereocontrolled total synthesis of gibberellin A_{15} and the formal total synthesis of gibberellin A_4. Many partial syntheses of gibberellins have also been published[42,43].

(a) Total Synthesis

Nagata et al[40] described the stereocontrolled total synthesis of gibberellin A_{15} [XXXI] (a C_{20}-GA) in the racemic form. They started with the intermediate enone [XXXII]. This already had the correct stereochemistry for the A, B and the lactone rings. The N-mesyl piperidine ring was expected to be stable to further elaboration and to be readily converted to the lactone.



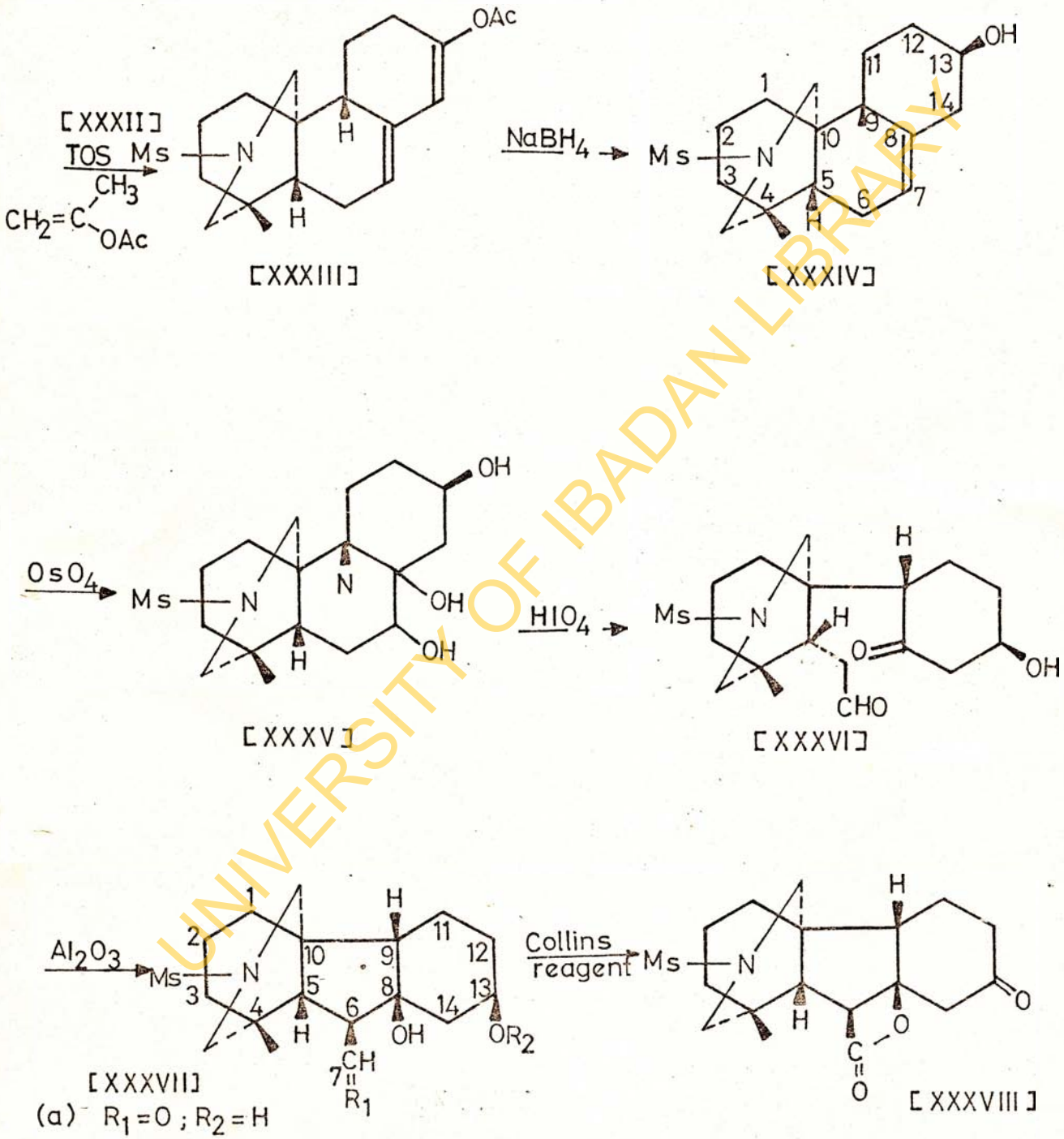
[XXXI]



[XXXII]

The first stage in the synthesis was the contraction of ring B. This was carried out as follows: The enone starting material [XXXII] was refluxed with isopropenyl acetate in the presence of p-toluenesulphonic acid, yielding the dienol acetate [XXXIII] as the major product. This was reduced with sodium borohydride in alkaline medium giving the hydroxy olefin [XXXIV] as the main product. Oxidation of [XXXIV] with osmium tetroxide gave the triol [XXXV] which gave the ketoaldehyde [XXXVI] on oxidation with periodic acid. The ketoaldehyde was cyclised in contact with neutral alumina to give the tetracyclic aldehyde [XXXVIIa].

SCHEME 9

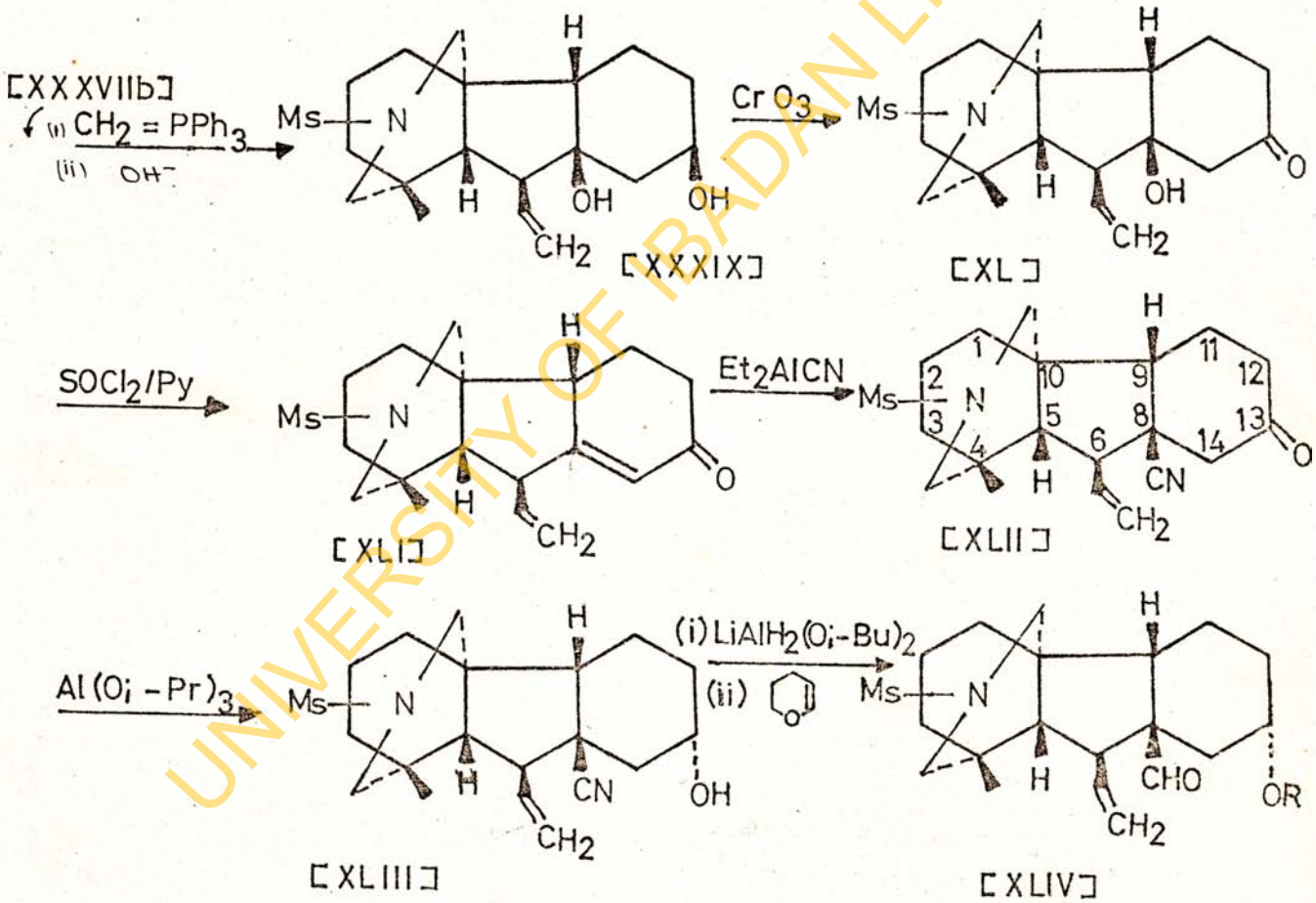


Configurations at C-6, C-8 and C-9 of [XXXVIIa] were assigned on the following reasons. It was believed that the stereochemistry at 9 was retained throughout the transformation from [XXXIV] since no basic reagents which could have caused epimerization were used. For the determination of the configurations of the 8-hydroxyl and the 6-formyl group, some derivatives, [XXXVIIb], and [XXXVIIc] were prepared and their infra-red spectra measured. The IR results indicated that in [XXXVIIb], the 8-hydroxyl was hydrogen bonded to the 13-β acetoxyl and the acetal oxygen in [XXXVIIc]. It also showed that the 13-β hydroxyl was hydrogen bonded to the 8-hydroxyl in [XXXVIIc]. This showed that all the substituents at C-6, C-8 and C-13 are oriented cis and therefore β. Decisive evidence for the β configuration of the formyl group attached to C-6 was provided by the fact that mild oxidation with chromic anhydride-pyridine complex in methylene chloride (Collins reagent) gave the β lactone [XXXVIII].

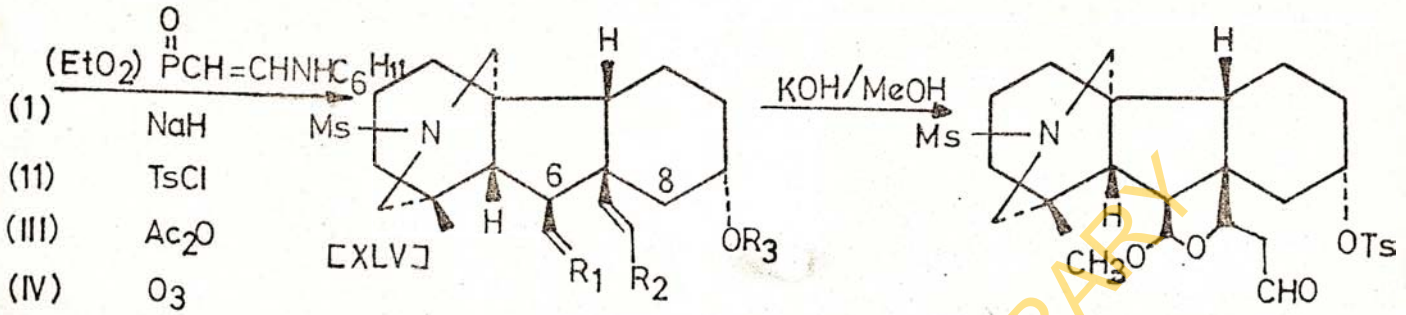
The next stage in the synthesis was the construction of the D ring. Compound [XXXVIIb] on Wittig vinylation followed by alkaline hydrolysis gave [XXXIX]. Jones oxidation of [XXXIX] gave [XL]. This was converted to the dienone [XLI] by dehydration with thionyl chloride in methylene chloride - pyridine at -73°. The dienone was then hydrocyanated by treatment with an excess of diethyl aluminium cyanide[44] in methylene chloride at room temperature giving the cis-

cyano ketone [XLII]. The β configuration was assigned to the C-6 vinyl group because the IR spectrum of [XL] indicated that hydrogen bond occurred between the C-6 vinyl and the 8-hydroxyl showing that they are cis. No epimerization was considered to have occurred at C-6 during the transformation of [XXXIX] to [XLII].

SCHEME 10



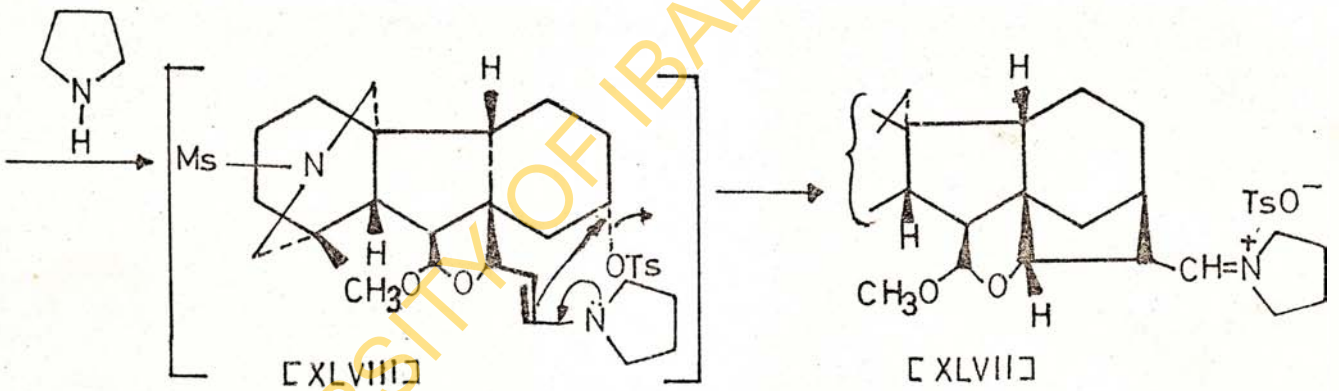
Scheme 10 contd.



[XLV]

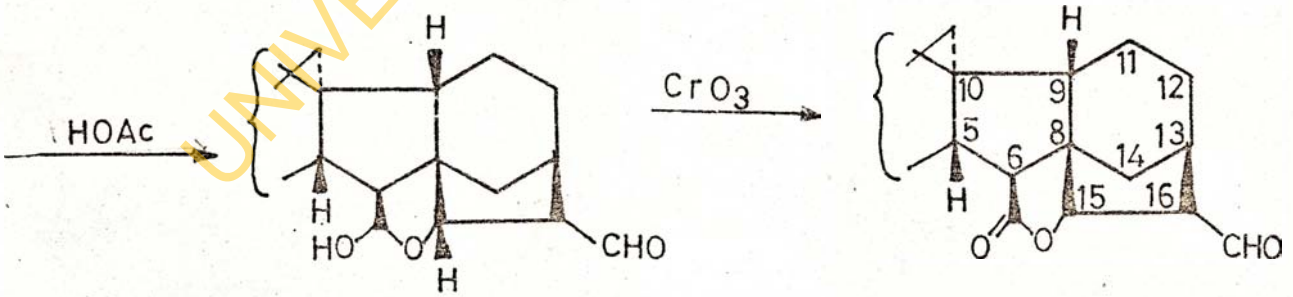
[XLVI]

	R_1	R_2	R_3
(a)	CH_2	CHO	H
(b)	CH_2	CHO	Ts
(c)	CH_2	$CH(OAc)_2$	Ts
(d)	O	$CH(OAc)_2$	Ts



[XLVIII]

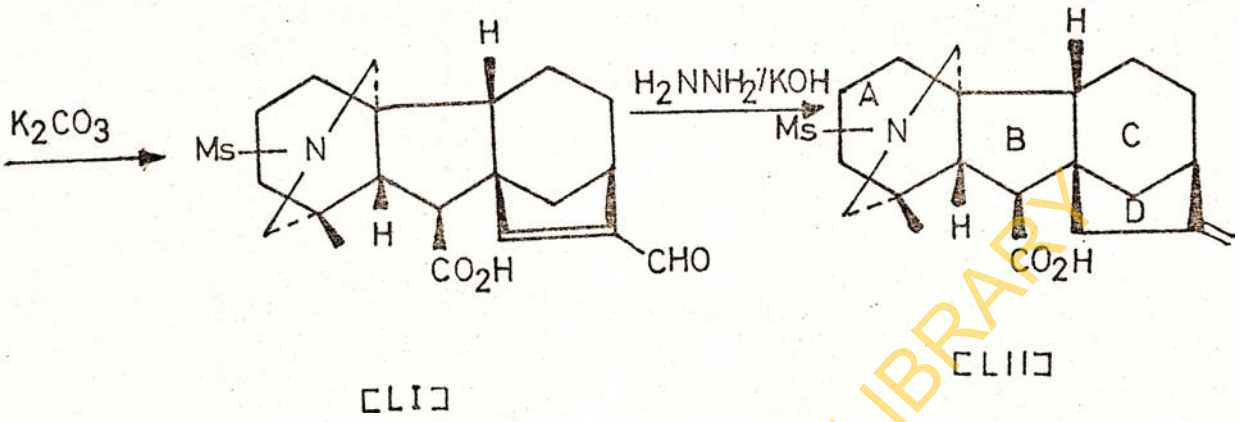
[XLVII]



[XLIX]

[L]

Scheme 10 contd.



[LI] [LII] [LIII]

The next stage in the construction of ring D was the conversion of the 13-keto group in [XLII] into a suitable α-oriented leaving group. Reduction of [XLII] with aluminium isopropoxide gave the alcohol [XLIII] as a main product. Its 13-epimer was a minor product. The angular cyano group in [XLIII] was converted to a formyl group by reduction with diisobutyl lithium aluminium hydride followed by hydrolysis with aqueous acetic acid and sodium acetate in THF giving the hydroxyaldehyde [XLIVa]. The 13-hydroxyl group in [XLIVa] was protected with dihydropyran giving the tetrahydropyranyloxyaldehyde [XLIVb]. Formyl olefination of [XLIVb] by treatment with sodium diethyl β-(cyclohexylamino) vinylphosphonate[45] in dry THF followed by hydrolysis with aqueous oxalic acid and 10% perchloric acid gave the formyl olefin [XLVa]. The hydroxyformyl olefin was then converted to the tosylate [XLVb] by treatment with

p-toluenesulphonyl chloride, thus providing a suitable leaving group at the 13 α-position.

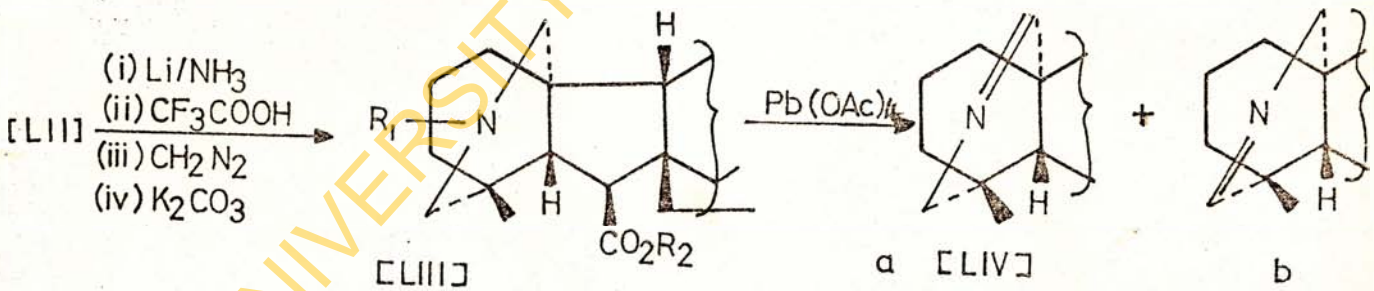
Attempts to selectively cleave the 6-vinyl group of [XLVb] to the formyl group by ozonolysis proved unsuccessful because of the apparent higher reactivity of the formyl olefin side-chain at C-8. The formyl group was deactivated by acetylation (The acetyl groups provided steric hinderance). The diacetate [XLVc] was prepared by reacting [XLVb] with acetic anhydride in methylene chloride in the presence of zinc chloride. Ozonolysis of [XLVc] gave the formyl derivative [XLVd].

Compound [XLVd] was cyclised to give the correct BCD skeleton as follows: On treatment of [XLVd] with potassium hydroxide in dry methanol and THF at -8° for 5 minutes, [XLVI] was obtained. What happened was the hydrolysis of the acetate groups, conversion of the C-6 formyl group to a hemi-acetal and the Michael addition of the hemi-acetal to the C-8 formyl olefin double bond. [XLVI] was treated with pyrrolidine in dry methanol and N-methylpyrrolidone at room temperature overnight and then at 70° for 1.5 hours giving [XLVII] through the intermediate enamine [XLVIII]. The cyclization was interpreted as an intramolecular SN_2 reaction of the intermediate enamine [XLVIII]. [XLVII] was hydrolysed by heating with 50% acetic acid at 100° for one hour giving the hexacyclic formylhemiacetal [XLIX]. Treatment of [XLIX] with Collins reagent effected the

selective oxidation of the hydroxyl group giving a mixture of the formyl lactone [L] epimeric at C-16. The lactone ring was opened by treatment with aqueous potassium carbonate giving the carboxylic acid [LI] with the loss of the asymmetric centres at C-15 and C-16. Wolff-Kishner reduction of [LI] was accompanied by endo/exo isomerisation of the double bond giving the exo-methylene-carboxylic acid [LII]. The correct B/C/D skeleton was thus achieved.

The final stage of the synthesis involved the removal of the N-mesyl protecting group to give the piperidine ring which was ultimately converted to the δ-lactone. This was effected according to the following scheme. Reductive elimination of the mesyl group

SCHEME 11

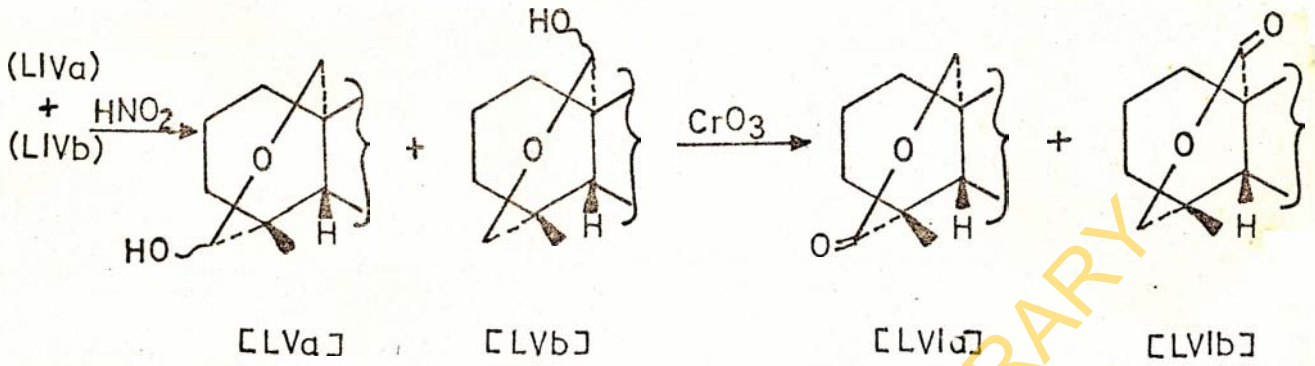


[LIII]

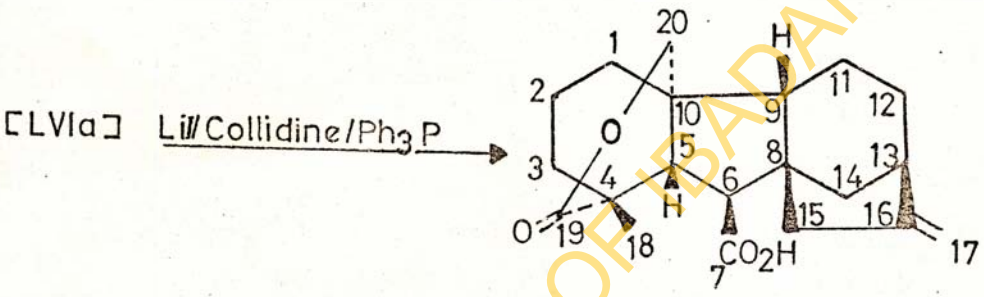
[LIV]

	R_1	R_2
(a)	H	H
(b)	CF_3CO	H
(c)	CF_3CO	CH_3
(d)	H	CH_3

Scheme 11 contd.



[LVa] [LVb] [LVIa] [LVIb]



[XXXI]

was achieved by treating [LII] with lithium in liquid ammonia in the presence of tert-butyl alcohol as a proton source, giving compound [LIIIa], isolated as the hydrochloride. (Simultaneous reduction of the carboxyl group occurred to a negligible extent). In other to selectively methylate the carboxyl group, the secondary amino group was first protected as the trifluoroacetylamide [LIIIb]. Methylation

of [LIIIb] with diazomethane gave the methyl ester [LIIIc]. The trifluoroacetyl protecting group was selectively removed by refluxing the methanol solution of [LIIIc] with 3N K_2CO_3 giving [LIIId].

The piperidine ring was converted to the δ-lactone by the following method: [LIIId] on dehydrogenation with lead tetraacetate gave a mixture of the isomeric azomethines [LIVa] and [LIVb]. The mixture on treatment with nitrous acid gave a mixture of isomeric hemiacetals [LVa] and [LVb]. The mixture was oxidized with Collins reagent giving a mixture of the two isomeric lactones [LVIa] and [LVIb]. They were separated by preparative thin-layer chromatography and the desired lactone, [LVIa] which was dl-gibberellin A_{15} methyl ester was obtained pure.

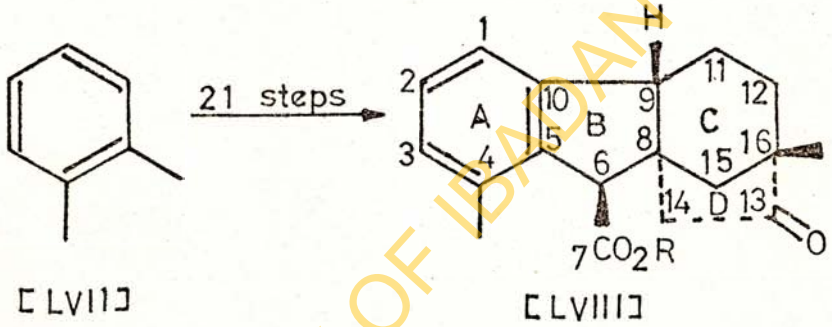
The methyl ester was demethylated by refluxing it in collidine with lithium iodide and triphenylphosphine for one hour. This gave the acid, dl-GA_{15} [XXXI] mp. 236-237°. The acid was proved to be a racemic form of GA_{15} by comparing its spectral data with those of an authentic sample of gibberellin A_{15}.

Another total synthesis of gibberellin that has been published was the formal total synthesis of gibberellin A_4 [XIV] (a C_{19} - gibberellin) by Mori and co-workers[41]. This synthesis can be divided into five stages.

The first stage involved the synthesis of epigibberic acid [LVIIIa] from o-xylene [LVII] through twenty-one steps[46].

The second stage involved the activation of ring A of epigibberic acid so that the required functional groups could be introduced. This was accomplished by the following scheme. Nitration of racemic epigibberic acid methyl ester gave the nitro ester [LIX]. This nitro ester gave the amino ester [LX] on hydrogenation over 10% palladium - charcoal catalyst. Diazotization of the amino ester

SCHEME 12



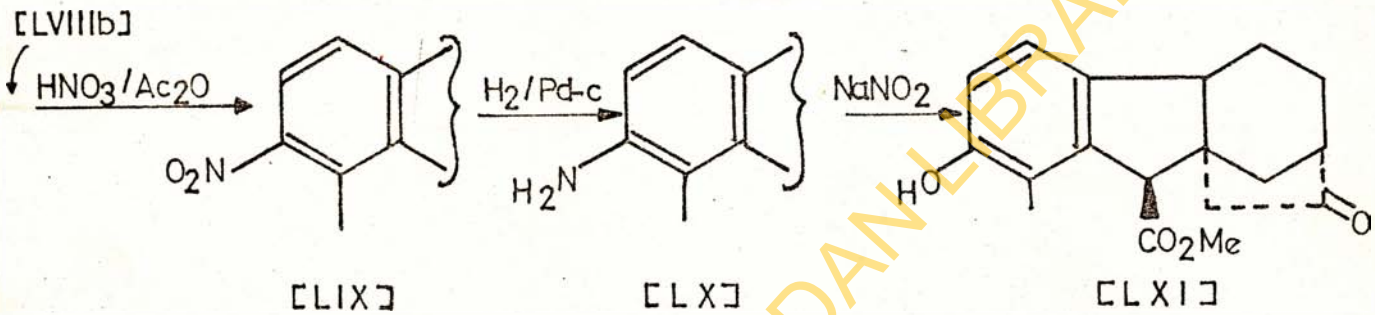
[LVII] [LVIII]

(a) R = H

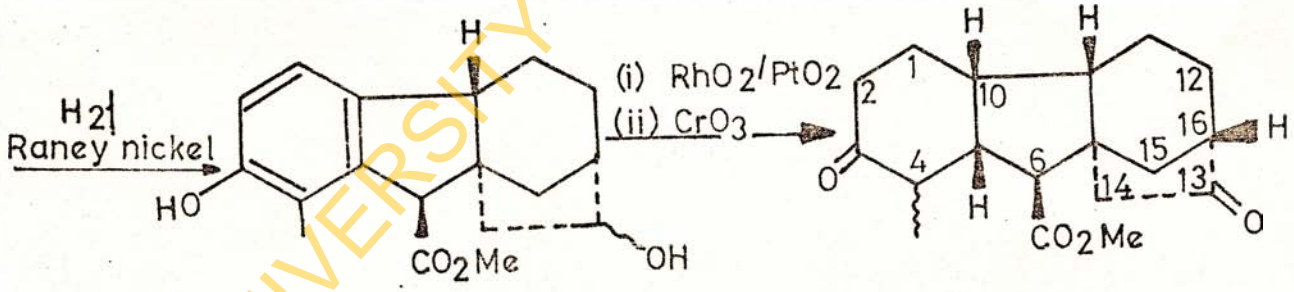
(b) R = CH_3

followed by hydrolysis gave the hydroxy ester (LXI). Hydrogenation of this ester over Raney nickel resulted in the reduction of the C-13 carbonyl group giving the dihydroxy ester (LXII]. Further hydrogenation of this dihydroxy ester over rhodium-platinum oxides gave a complex mixture of esters in which the A ring had been completely hydrogenated. The mixture was oxidised with Jones' reagent, and the racemic ester [LXIII] was isolated as one of the products.

SCHEME 13

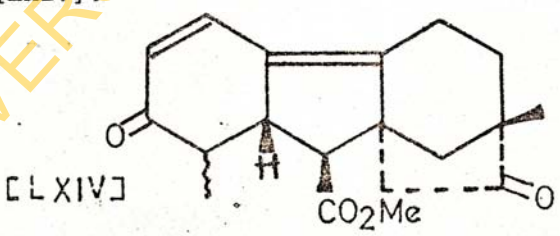


[LIX] [LX] [LXI]



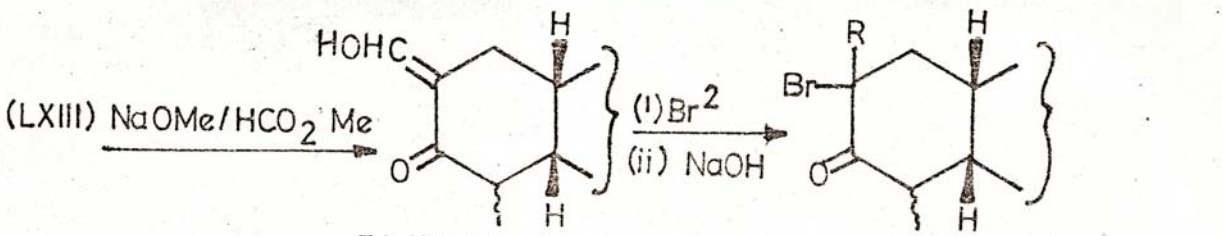
[LXII] [LXIII]

The third stage involved the conversion of the dioxo ester [LXIII] to the dienone [LXIV]. In order to introduce a double bond into the 1,2 position, an α-formyl group was used as an activating group for the introduction of a bromine atom. Treatment of [LXIII] in dry THF with sodium methoxide in dry benzene and methyl formate gave on work-up the formyl ketone [LXV] which was brominated to give the bromide [LXVI]. Decarbonylisation of the bromide [LXVI] with sodium hydroxide gave the bromoketone [LXVII]. This was dehydrobrominated to give an α-β unsaturated ketone [LXVIII]. Boiling dilute hydrochloric acid isomerised [LXVIII] to the β, γ-unsaturated ketone, [LXIX]. Migration of the double bond was effected by treating [LXIX] with 10% palladium-charcoal to give [LX]. In order to introduce the 1,2-double bond, the diketone, [LXX] was successively brominated and dehydrobrominated according to the sequence described above for the dioxo ester [LXIII], ultimately yielding the dienone [LXIV].



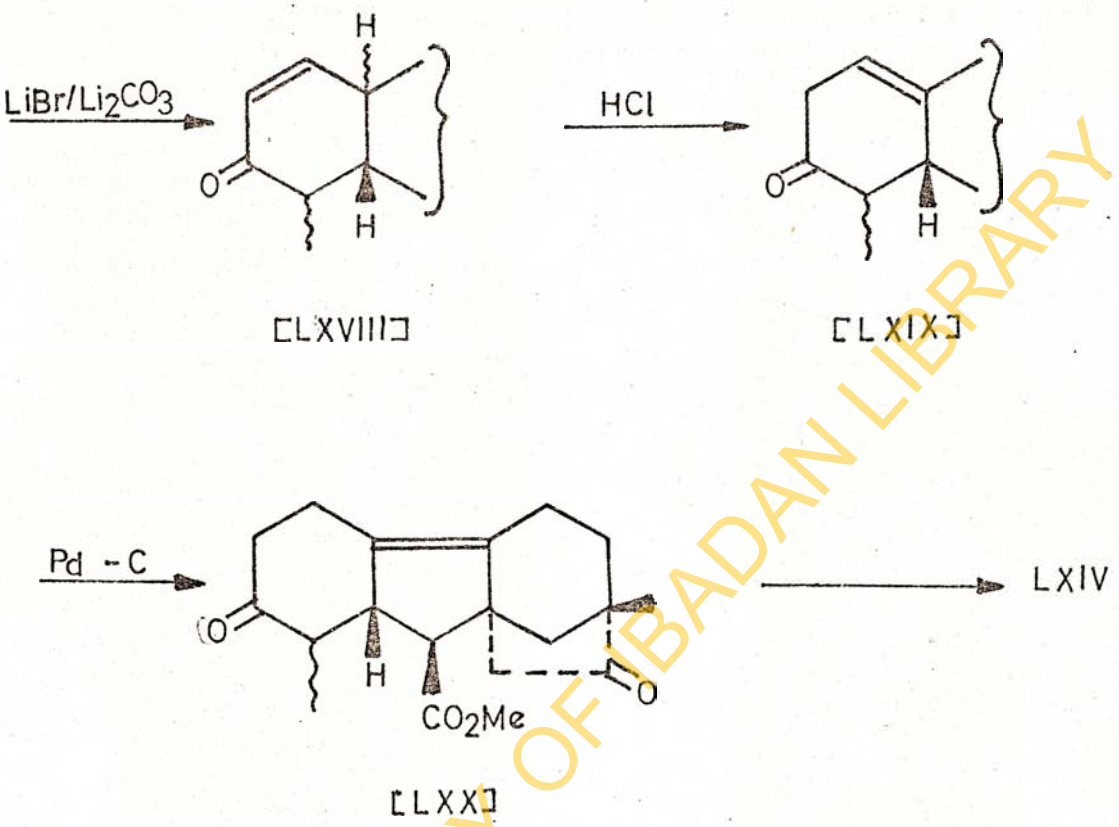
[LXIV]

SCHEME 14



[LXV] [LXVI] R = CHO, [LXVII] R = H

Scheme 14 contd.



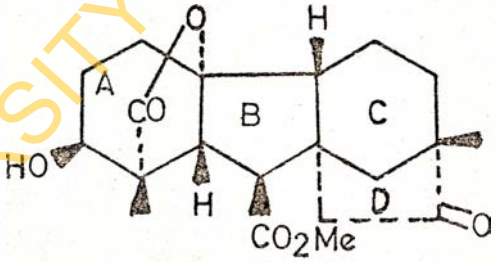
[LXVIII] [LXIX]

[LXX]

The fourth stage involved the partial synthesis of the methyl ester, [LXXI] from the dienone [LXIV]. This essentially led to the formation of the ring A lactone and the introduction of the 3-hydroxyl group.

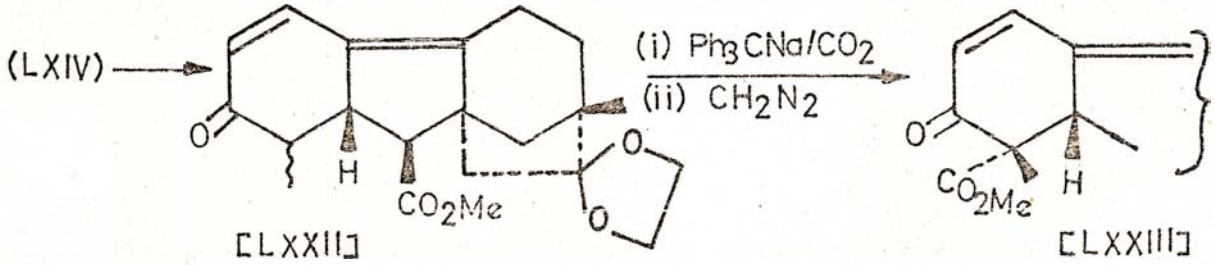
The dienone [LXIV] was ketalized with excess ethylene glycol and p-toluene sulphonic acid in boiling dichloroethane giving the monoketal [LXXII]. In order to introduce the lactone bridge in

ring A an α-oriented carboxyl group had to be attached to C-4, of [LXXII]. This carboxylation was carried out with ethereal triphenylmethyl sodium and carbon dioxide. The resulting mixture was esterified with diazomethane after acidification, giving a mixture of esters from which the diester monoketal [LXXIII] was isolated. The reduction of the diester monoketal with sodium borohydride gave the hydroxy ester [LXXIV]. Hydrogenation of the hydroxy ester [LXXIV] over palladium-charcoal gave [LXXV]. [LXXV] was lactonised and deketalised by boiling with dilute sulphuric acid and the product treated with diazomethane giving the hydroxyester [LXXVI]. The 3-hydroxyl group of [LXXVI] was epimerised by treatment with dilute aqueous sodium hydroxide giving the methyl ester [LXXI] on work-up.



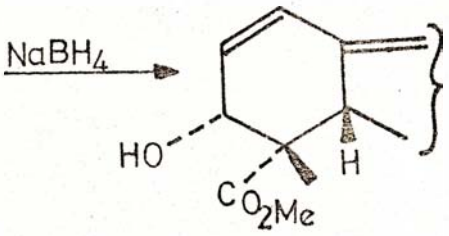
[LXXI]

SCHEME 15

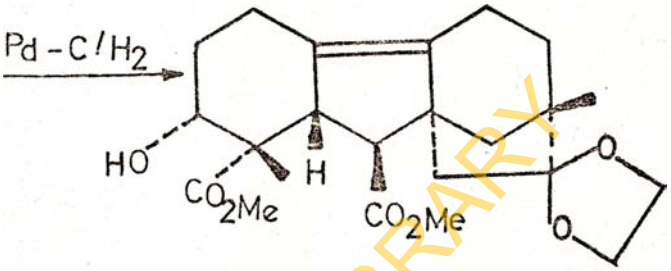


[LXXII] [LXXIII]

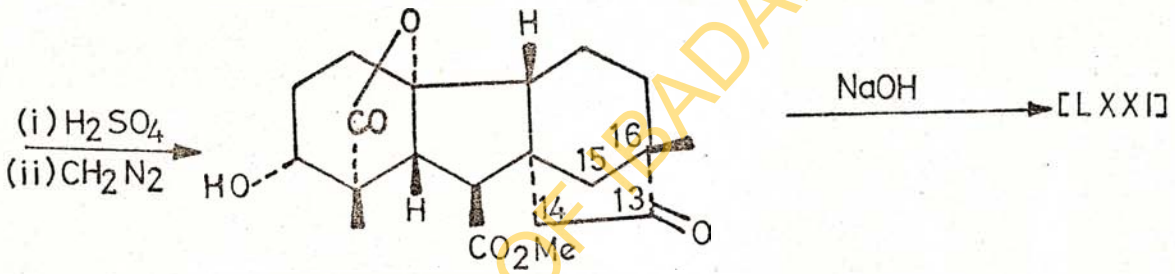
Scheme 15 contd.



[LXXIV]



[LXXV]



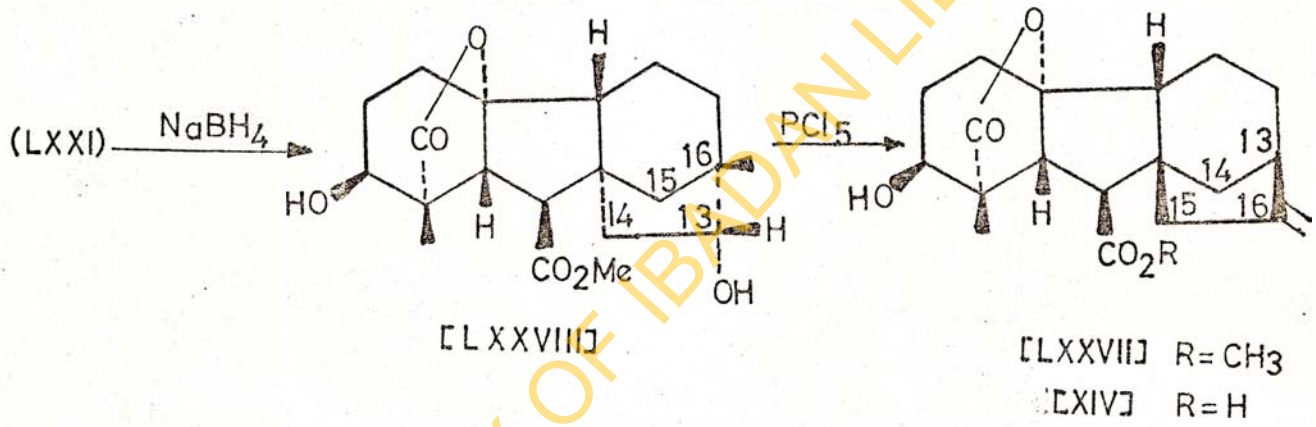
[LXXV]

[LXXI]

The fifth and final stage of the synthesis involved the rearrangement of the C/D rings. Cross et al[47] had converted the methyl ester, [LXXI] to gibberellin A_4 methyl ester [LXXVII) by the following process. They reduced [LXXI] with sodium borohydride giving the diol [LXXVIII]. This was then rearranged to gibberellin A_4

methyl ester [LXXVII] by treatment with phosphorus pentachloride. Mori et al[41] then demethylated the gibberellin A_4 methyl ester by boiling with dilute aqueous sodium hydroxide to give gibberellin A_4 [XIV] and its 3-hydroxy epimer.

SCHEME 16



[LXXVIII]

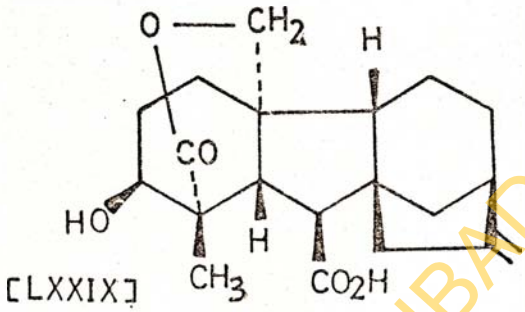
[LXXVII] R = CH_3

[LXIV] R = H

(b) Partial synthesis

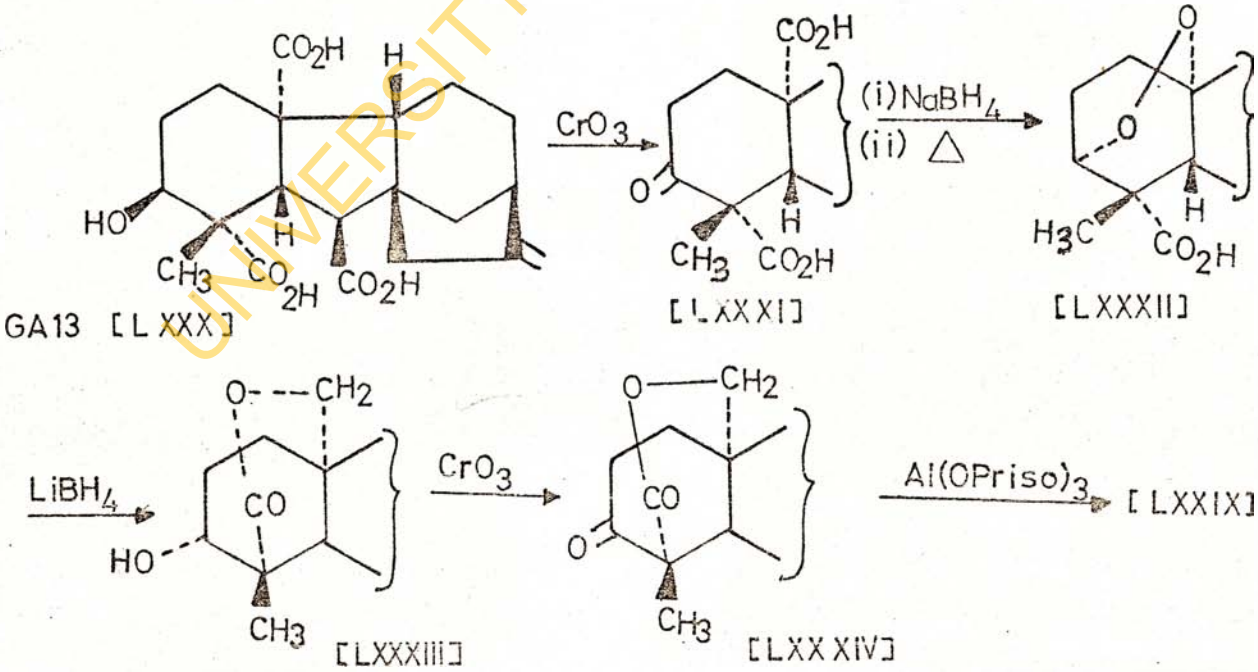
Some partial synthesis of gibberellins have been described in literature, for example the partial synthesis of gibberellin A_{37} [LXXIX] from GA_{13}[42] [LXXX]. GA_{13} was oxidised with Jones reagent to give the 3-keto derivative [LXXXI] which was then converted to the 20,3-lactone [LXXXII] by reduction with sodium borohydride followed by heating at 135°. Reduction of the lactone with lithium borohydride gave the 3-epimer [LXXXIII] of GA_{37}. The synthesis of

the lactone [LXXXII] enabled the selective reduction of the 10-carbonyl group of GA_{13} [LXXX]. This carbonyl group is the most hindered out of the three carbonyl groups in GA_{13} and therefore the least susceptible to reduction. The 3-epimer [LXXXIII] of GA_{37} was then converted into GA_{37} [LXXIX] by oxidation to the 3-ketone [LXXXIV] with Jones' reagent and subsequent reduction with aluminium isopropoxide in isopropanol.

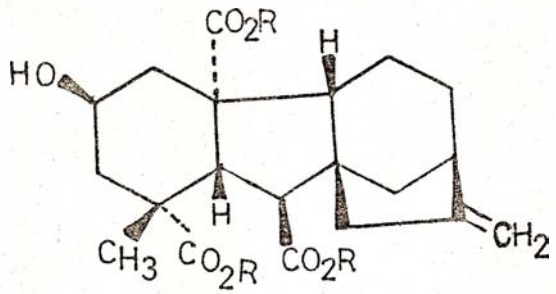


[LXXIX]

SCHEME 17

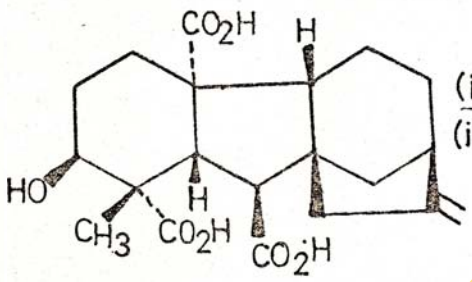


A general method for the partial synthesis of 2-hydroxy gibberellins from the more abundant 3-hydroxy gibberellins has been described[43]. The structure of GA_{46} [LXXXVa] was established by this method by partial synthesis from gibberellin A_{13} [LXXX]. GA_{13} was treated with sodium metaperiodate and osmium tetroxide, followed by methylation with diazomethane giving the nor-ketone methyl ester [LXXXVI]. On dehydration with phosphorus oxychloride in pyridine, [LXXXVI] gave the olefin [LXXXVII]. The olefin was converted to the bromohydrin [LXXXVIII] on treatment with acetyl hypobromite. The hydroxyl group in [LXXXVIII] was protected as the trimethylsilyl ether and this derivative was then debrominated with tri-n-butyltin hydride giving the 2-alcohol as the trimethyl silyl ether [LXXXIX]. [LXXXIX] was subjected to the Wittig reaction, thus converting it to [XC]. This on oxidation with Jones reagent gave the ketone [XCI]. Reduction of this ketone with aluminium isopropoxide prepared from aluminium and propan-2-ol in the presence of mercury[II] chloride and carbon tetrachloride gave GA_{46} methyl ester [LXXXVb] and 10% of the 2α-isomer.

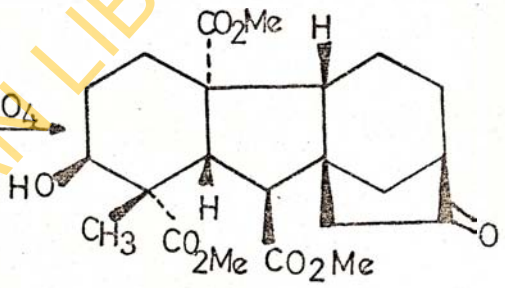


[LXXXVa]

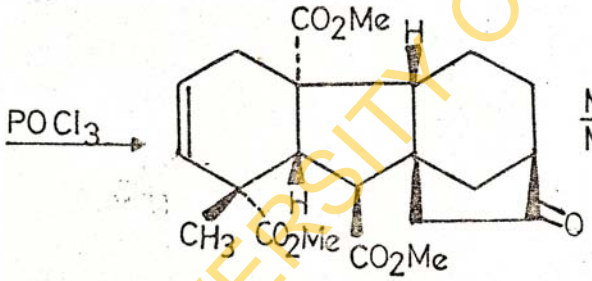
SCHEME 18



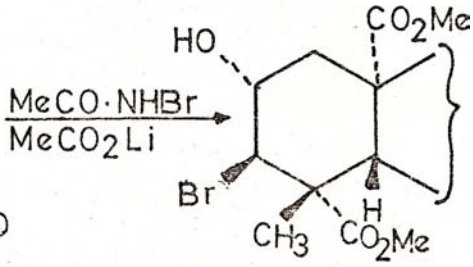
[LXXX]



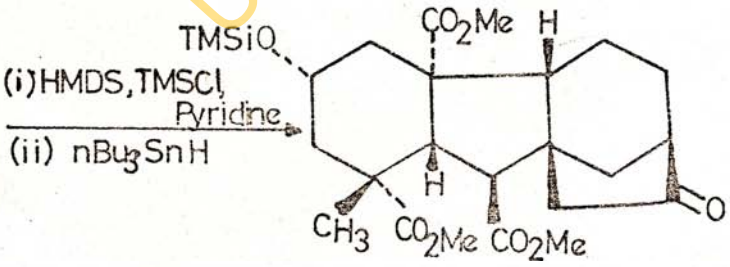
[LXXXVI]



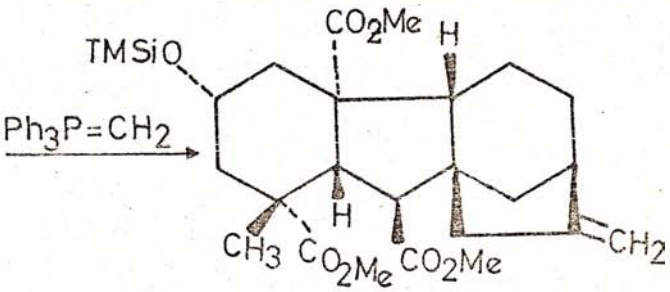
[LXXXVII]



[LXXXVIII]

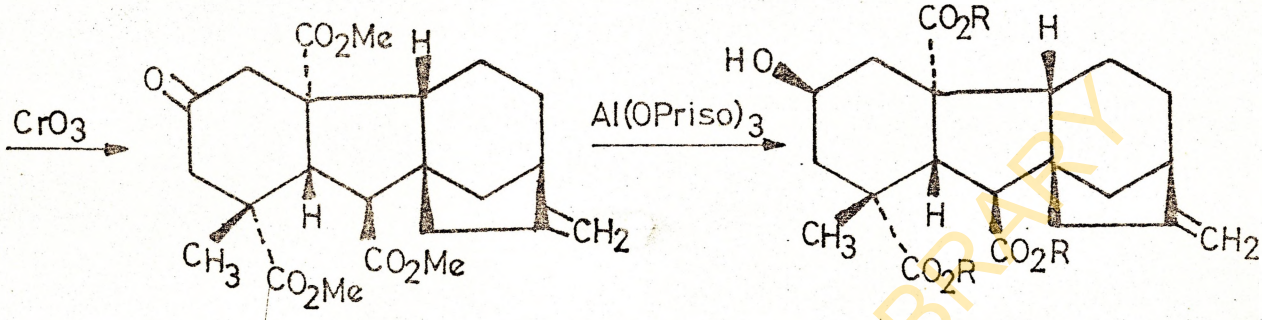


[LXXXIX]



[XC]

Scheme 18 contd



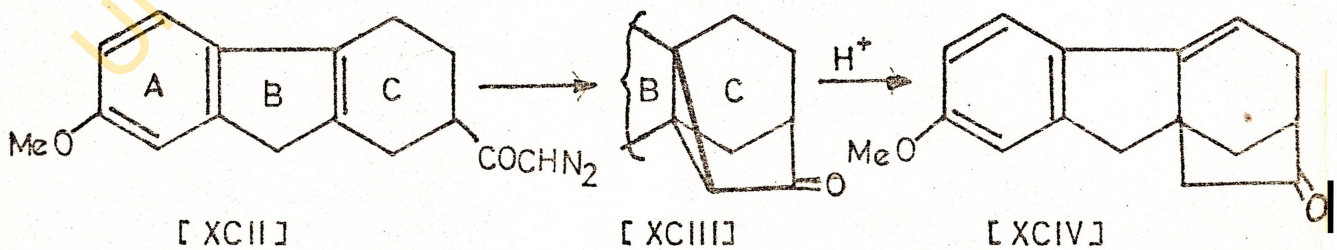
[XCI]

(LXXXVb) R = CH_3

(LXXXVa) R = H

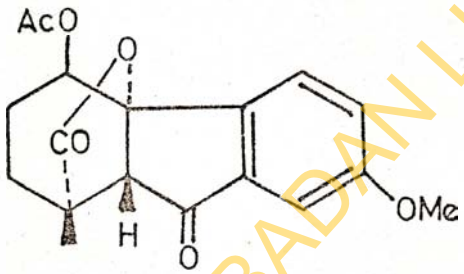
Several other approaches to the synthesis of the gibberellins have been described. One of these, described by Dasgupta et al[48] involved intramolecular carbene insertion reaction. The diazoketone [XCII] was cyclised to the cyclopropane derivative [XCIII]. This on acid-catalysed cleavage of the cyclopropane ring gave [XCIV] which has the basic skeleton of the gibberellins.

SCHEME 19



[XCII] [XCIII] [XCIV]

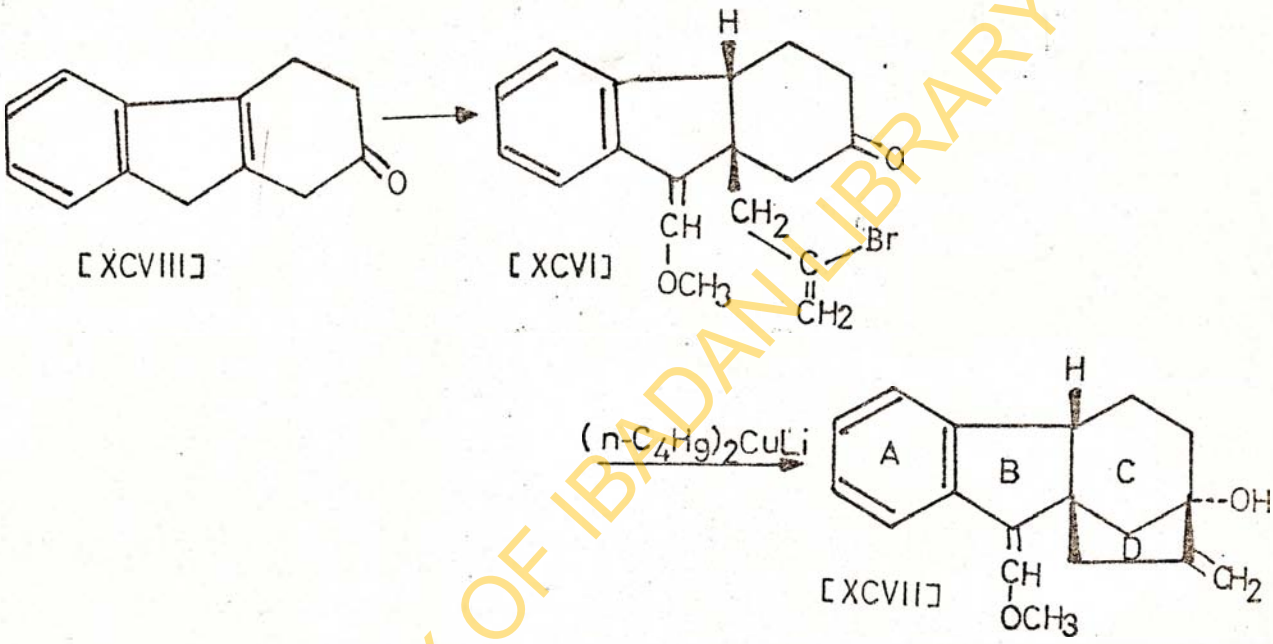
Another approach was the synthesis of the hydrofluorenone derivative [XCV] by Hori and Nakanishi[49]. This was synthesized in high yield in several steps from ethyl pyruvate and malononitrile. This compound [XCV] was believed to be a possible intermediate in the total synthesis of the C_{19}-gibberellins.



[XCV]

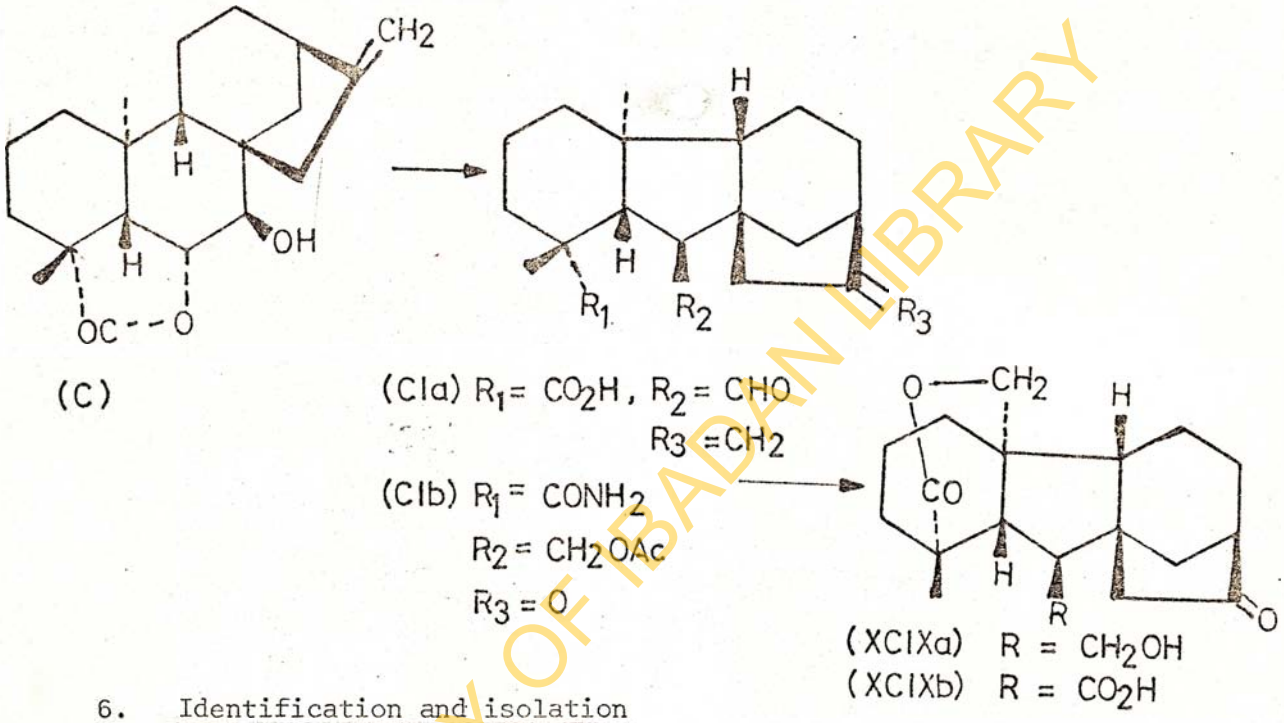
One interesting approach described by Corey et al[50] had as its key step the construction of the D ring of the gibberellin skeleton by intramolecular reductive addition of a δ- or ε-halo ketone in the presence of an appropriate organometallic reagent. Thus the reaction of [XCVI] with 6 equivalents of di-n-butyl-copperlithium in ether (0.15M) at -50° for 2.5hr gave [XCVII] in 73% yield. [XCVI] had been prepared from the tricyclic ketone [XCVIII]

SCHEME 20



Another interesting approach was the partial synthesis of gibberellin A_{15} norketone [XCIXb] from 7-hydroxykaurenolide(C) by Cross and Gatfield[51]. 7-hydroxykaurenolide(C) was transformed[52,53] to the aldehydo-acid (Cla) which was then converted to the amide (Clb). The amide was photolysed in benzene in the presence of lead tetra-acetate and iodine, giving the lactone [XCIXa] which on Jones oxidation gave gibberellin A_{15} norketone [XCIXb].

SCHEME 21



6. Identification and isolation

The gibberellins occur at very low endogenous levels in plants. The methods used for the detection, identification, and isolation of the gibberellins are therefore very sensitive. Extensive purification of extracts are normally required. The extraction procedure normally used for the determination of the gibberellin content of plant materials is summarised in the scheme below:

Plant materials are homogenised with 80% aqueous methanol in a blendor.

↓

The homogenised material is filtered and the residue further extracted twice with 80% aqueous methanol.

↓

The combined filterates are concentrated under reduced pressure to remove all the methanol.

↓

The pH of the aqueous layer is adjusted to 8.0 and the aqueous layer is then extracted three times with petroleum ether (60^{o}-80^{o}). The petroleum ether extract is discarded.

↓

The aqueous layer is then extracted thrice with ethyl acetate giving the neutral ethyl acetate (NE) fraction.

↓

The pH of the aqueous layer is then adjusted to 3.0 and the aqueous layer is extracted three times with ethyl acetate. This is the acidic ethyl acetate (AE) fraction.

↓

The aqueous layer is then extracted with n-butanol giving the acidic n-butanol (AB) fraction.

↓

The aqueous layer is discarded.

The neutral ethyl acetate (NE) fraction contains the gibberellin glucosyl esters. Most free gibberellins are extracted in the acidic ethyl acetate (AE) fraction. The acidic butanol (AB) fraction contains the gibberellin glucosides and some polar gibberellins, such as gibberellins A_{32} and A_{28}.

Some of the methods used for purifying and separating the gibberellins further are counter-current distribution[54,55], column chromatography[56], paper chromatography[57], gel-filtration[58] and thin-layer chromatography[59,60]. The gibberellins can be conveniently viewed under U.V. light on thin-layer chromatography plates[59] after spraying with ethanol/concentrated sulphuric acid (95:5) and heating at 120° for 10 minutes.

Gas chromatography has been extensively used[60] in the separation and identification of gibberellins since it was first applied by Ikekawa et al in 1963[61].

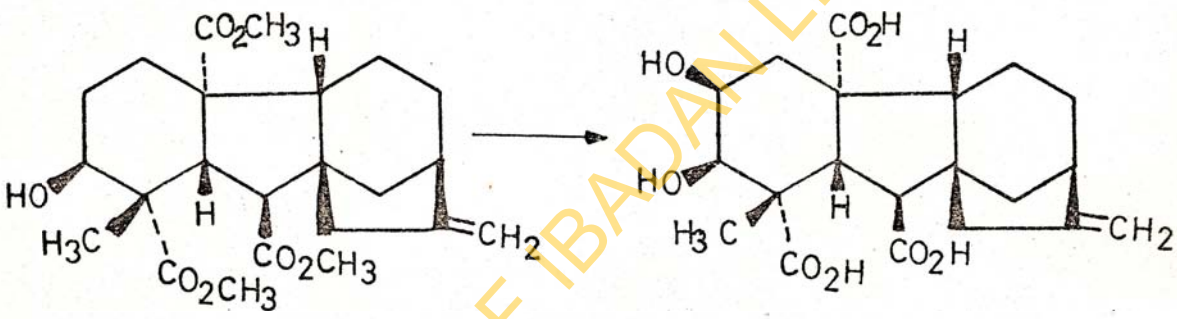
The most powerful tool in the identification of gibberellins is the combined gas chromatography - mass spectrometry (GC-MS)[62,63]. It is a very sensitive and conclusive method of identification of the

various known gibberellins without the need of reference compounds This is because reference mass spectra of the methyl esters and the trimethylsilyl ethers of the methyl esters (where appropriate) of the known gibberellins are available[39,64]. On account of its sensitivity it requires little or no purification where the concentration of the endogenous gibberellins in the extract is relatively high (for example greater than one per cent). Extensive purification is however still necessary with very low concentrations. It has also proved very useful in the detection and identification of new gibberellins[65,66]. This is possible because the mass spectrum of any new gibberellin will feature the characteristic gibberellin-like fragmentation patterns which were discussed earlier. It will however be distinguishable from the known gibberellins. It may be possible to suggest a structure for it which can then be confirmed by partial synthesis and nuclear magnetic resonance.

An example of the application of these methods is provided by the detection and characterization of GA_{43}[66] [C11]. This gibberellin was detected as a new gibberellin in the endosperm of Echinocystis macrocarpa by GC-MS. The mass spectrum of its MeTMSi derivative (M^+ at 580 a.m.u.) exhibited the fragmentation patterns characteristic of gibberellins[39], but could be distinguished from those of the known gibberellins. The mass spectrum had prominent M^+-90, M^+-91, and M^+-92 ions associated[39] with the presence of at least two methoxycarbonyl

groups. Peaks at m/e 147[39], and 217[55], characteristic of 2,3-dihydroxy gibberellins were also present. The structure [CII] was tentatively assigned to it and this was later confirmed by partial synthesis from GA_{13} trimethyl ester [CIII].

SCHEME 22



[CIII] [CII]

Several biological assays[67,68] have also been developed for the detection of the gibberellins. The most common of these make use of the ability of gibberellins to promote growth extension in intact plants. Examples of these are the dwarf rice seedling bioassay[67], dwarf pea bioassay[67], and the lettuce hypocotyl bioassay[69]. In general the seeds are sown in a suitable medium and allowed to germinate for an appropriate period. The test solution is then applied and after an appropriate period, the increase in length of a specific part of the

seedling is measured. The parts that are measured are the leaf sheath in the dwarf rice seedling bioassay, the shoot in the dwarf pea bioassay and the hypocotyl in the lettuce seedling bioassay.

Another important bioassay for gibberellins is the barley endosperm test developed by Nicholls and Paleg[70]. This test makes use of the ability of gibberellins to induce the production of α-amylase. The α-amylase accelerates the conversion of the starch in the endosperm to reducing sugar.

$$\underset{\text{starch}}{(C_6H_{10}O_5)n} + nH_2O \xrightarrow{\text{amylase}} \underset{\text{glucose}}{nC_6H_{12}O_6}$$

This test is insensitive to the other growth promoting hormones. Barley grains are cut transversely into two parts, and the embryo-less, endosperm part is placed in the test solution. An antibiotic (streptomycin) is normally added to the test solution to prevent the growth of bacteria. The mixture is then usually incubated for two days at 30°C. The amount of reducing sugar released from the endosperm into the solution is then quantitatively determined[71].

7. Chemistry

The chemistry of the gibberellins has been studied in the course of their structural elucidation. Because of the similarity in the structures of the various gibberellins, many of them undergo some similar general chemical reactions. On the other hand the various structural variations of the different gibberellins also affect the

course of some of the important chemical reactions, that are encountered in gibberellin chemistry.

Ring A of the gibberellins is labile to strong base. If there is no double bond in the 1,2-position, epimerization of 3β-hydroxyl group occurs in dilute alkali to an equilibrium mixture with the 3α-hydroxyl[72]. In the presence of a 1,2-double bond however, the 19 → 10-lactone ring undergoes an allylic rearrangement to form a 19 → 2-lactone without epimerisation of the 3β-hydroxyl in dilute alkali[72].

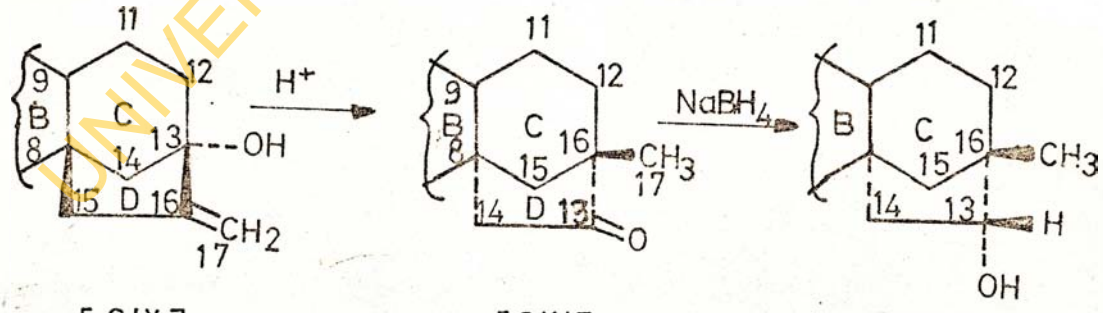
3β-hydroxyl groups are smoothly oxidized to 3-ketones only if the exocyclic methylene group had been reduced or removed[21]. The 3-ketones so obtained are reduced by alkali-metal hydrides to the 3α-epimer[72]. However if the reduction of the 3-ketone is effected with aluminium isopropoxide the 3β-hydroxyl is obtained as the major product[43].

3-hydroxyl groups can readily be dehydrated by reacting the gibberellin (usually as the methyl ester) with phosphorus oxychloride, to give the 2,3-dehydro compound. The dehydration can also be effected via the tosylate. The tosylate is prepared by reacting the gibberellin with p-toluene sulphonyl chloride in dry pyridine at room temperature. The resulting tosylate is converted to the 2,3-dehydro derivate by refluxing in collidine[73].

The 16-exocyclic double bond is readily cleaved by using sodium metaperiodate and catalytic amount of osmium tetroxide in pyridine giving the 16 nor-ketone[53].

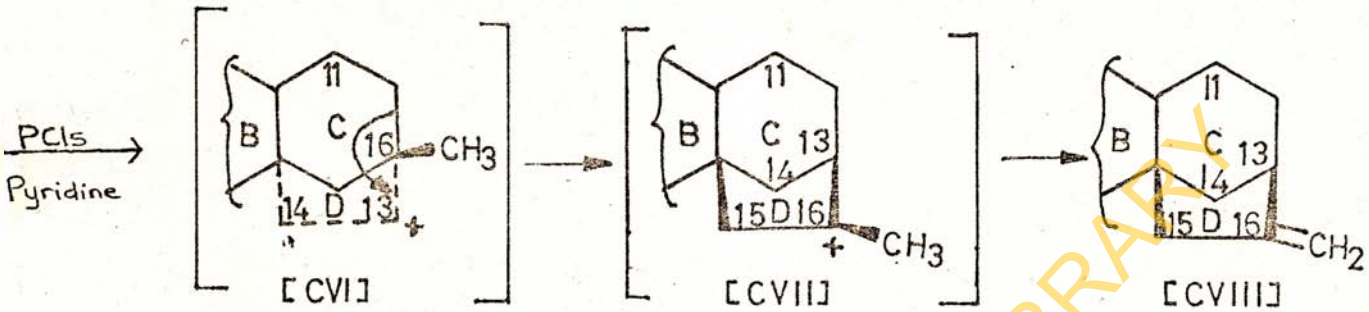
In the absence of a 13-hydroxyl group, the exocyclic methylene group is hydrated by treatment with mineral acid[31]. If a 13-hydroxyl group is present a Wagner-Meerwein rearrangement occurs, and the resulting nor-ketone [CIV] has an opposite configuration (α) in ring D[18,47]. This nor-ketone [CIV] gives the 16-hydroxyl compound [CV] on reduction. If this alcohol is treated with phosphorus pentachloride, a reversal of the Wagner-Meerwein rearrangement occurs[47], probably via the intermediate ions [CVI] and [CVII] to give the 13-deoxygibberellin system [CVIII]. The 13-hydroxyl

SCHEME 23



[CIX] [CIV] [CV]

Scheme 23 contd.



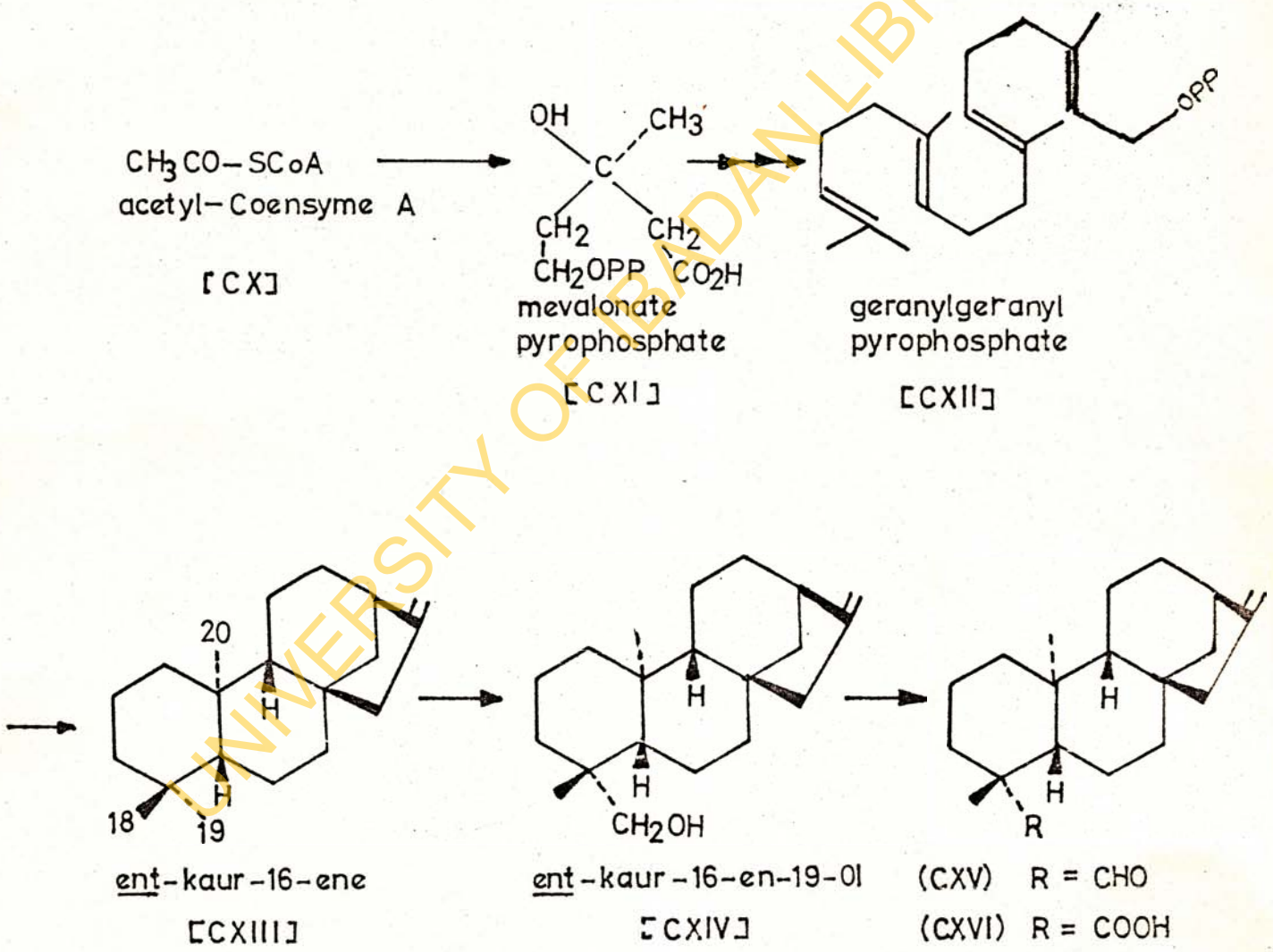
gibberellin system [CIX] can thus be converted to the 13-deoxygibberellin system [CVIII][47].

8. Biosynthesis and metabolism

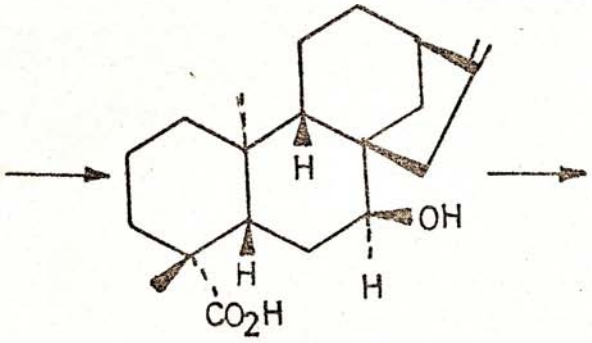
The biosynthesis and metabolism of the gibberellins have been studied in some detail both in the fungus Fusarium moniliforme and in higher plants[74.78]. It has been found that the early stages in the biosynthesis of the gibberellins follow the usual pathway for diterpenoids. This is from acetyl co-enzyme A [CX] via mevalonate pyrophosphate [CXI] to geranylgeranyl pyrophosphate [CXII]. The geranylgeranyl pyrophosphate is then cyclised to ent-kaur-16-ene [CXIII]. The ent-kaur-16-ene is oxidised through ent-kaur-16-en-19-ol [CXIV] and ent-kaur-16-en-19-al [CXV] to ent-kaur-16-en-19-oic acid [CXVI]. The acid is converted via ent-7α-hydroxykaur-16-en-19-oic

acid [CXVII] to ent-gibberellan-7-al-19-oic acid [CXVIII] (GA_{12} aldehyde). This is then converted to C_{20}-gibberellins, for example GA_{12} [CXIX] and C_{19}-gibberellins, for example GA_9 [CXX].

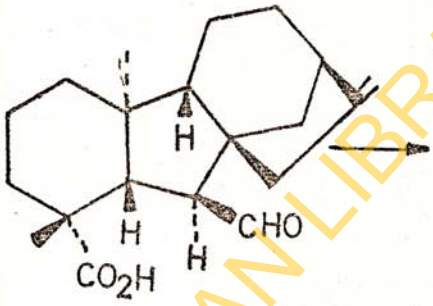
SCHEME 24



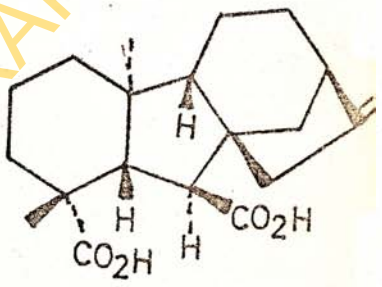
Scheme 24 contd.



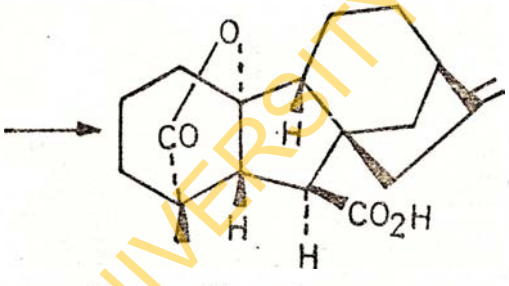
ent-7α-hydroxykaur-16-en-19-oic acid
[CXVII]



GA_{12}-aldehyde
[CXIII]



C_{20}-gibberellin (GA_{12})
[CXIX]



C_{19}-gibberellin (GA_9)
[CXX]

Most of the information about the biosynthetic pathway of the gibberellins was obtained through feeding experiments with labelled precursors to Gibberella fujikuroi and cell-free systems from some higher plants[74-78]. Thus various investigations, particularly by West and his group[78], based on the feeding of labelled mevalonate to Gibberella fujikuroi provided the information on the biosynthetic pathway of fungal gibberellins (Scheme 24).

Using cell-free systems of Echinocystis macrocarpa, West and his group[75] also showed that mevalonate was converted to ent-7α-hydroxy kaurenoic acid [CXVII] through ent-kaurene [CXIII], ent-kaurenol [CXIV], ent-kaurenal [CXV], and ent-kaurenoic acid [CXVI].

Graebe et al[76], working with cell-free system prepared from immature seed of Cucurbita pepo reported the conversion of mevalonic acid into gibberellin A_{12} aldehyde [CXVIII]. They fed labelled mevalonate to the system and one of the labelled products was GA_{12} aldehyde together with ent-kaurenoic acid [CXVI] and ent-7α-hydroxykaurenoic acid [CXVII].

Further feeding experiments by Graebe et al[77,78] using cell-free system from immature seeds of Cucurbia maxima provided evidence on the conversion of mevalonic acid to gibberellins in higher plants. They fed [^{14}C] mevalonic acid to the cell-free system and obtained [^{14}C] GA_{12}-aldehyde. They refed the labelled GA_{12}-aldehyde to the system and obtained the labelled C_{20}-gibberellins [^{14}C] GA_{13} and [^{14}C] GA_{43}

and a C_{19}-gibberellin [^{14}C] GA_4[77]. Thus it was shown that the pathway from mevalonic acid to the gibberellins was basically the same in the higher plants and in the fungus.

The pathway for the interconversion of the various GAs may however vary in different plants or even in different organs of the same plant. Normally any plant will produce only a few of the large number of gibberellins.

Hydroxylation and glucosidation of the GAs in any plant organ occur as the plant organ matures, although not all hydroxylations result in reduction of biological activity. 2β-hydroxylation is however a deactivation process and it occurs in all higher plants that have been studied. For example Frydman and MacMillan[79] reported that when fed to immature pea seeds, labelled GA_9 was metabolised to labelled 2β-hydroxy GA_9 (2-epi-GA_{40}). GA_{20} was also metabolised to GA_{29} (2β-hydroxyGA_{20}). It is believed that glucosides are biologically inactive storage forms, from which the free GAs are later regenerated during germination.

Three sites of biosynthesis of gibberellins have been identified in higher plants. These are immature seeds, root and shoot apices.

9. Uses

Some of the gibberellins have much use in agriculture[80] and industry[81]. In agriculture they are used to increase the yield and

quality of some fruits and in the preservation of others. Some examples are given below.

They are used for the following:

(a) regulating the time of harvest and increasing the yield and quality of grape fruits. The timing of the spraying of the crop with gibberellin solution is very important in this application;

(b) increasing fruit set in citrus, delaying the ripening of the fruits on the tree for extended period of many months without deterioration and preservation of the fruits during storage,

(c) preservation of banana fruits; and

(d) increasing yield in pear by spraying the trees at bloom.

Gibberellin A_3 (gibberellic acid) which is the most commercially available gibberellin is usually used for the various applications. GA_4/GA_7 mixture have also been used especially for reducing the usual June fruit drop.

In industry, the gibberellins particularly GA_3 have been used extensively in the malting stage of beer production[81].

The Auxins

The auxins are another group of plant hormones which are very effective among other things in promoting growth extension in plants.

They differ from the gibberellins however in that whereas they are very effective (when applied exogenously) in promoting growth extension of plant sections, they have little effect on intact plants[81a]. Gibberellins on the other hand promote large growth responses on intact plants but have little effect on plant sections.

The discovery of auxins stemmed from the observation of Charles Darwin in the last century on the bending of coleoptiles to unilateral light. This gave rise to a series of investigations which culminated in the isolation[82,83] of three compounds early in the century. They were reported to have growth promoting activity. They were called "auxin A", "auxin B" and "heteroauxin". "Auxin A" and "heteroauxin" were isolated by Kögl and co-workers[82,83] from human urine. "Auxin A" and "auxin B" have since been found to possess no growth promoting activity. Vliegenlbert and Vliegenlbert[84] using X-ray crystallography and mass spectrometry determined the structures of authentic samples of "auxin A" and "auxin B". They found that "auxin A" was cholic acid and "auxin B" was thiosemicarbazide.

The so called heteroauxin is indole-3-acetic acid (1AA) [CXXI], and it is now accepted as the most important natural auxin. The occurrence of 1AA has now been reported in a wide range of plants and plant tissues[85].

1. Physiological roles

The physiological role of auxins in plants is multifarious[86-88]. The major physiological effects include:

(a) promotion of growth extension in plants,

(b) promotion of the initiation of roots on cuttings,

(c) inducement of flowering and fruit set in some plants, for example pineapple and tomato,

(d) inhibition of the abscission of plant organs, such as leaves, flowers and fruits, and

(e) inducement of the syntheses or activities of some enzymes, for example they enhance the formation of cellulose synthetase in coleoptiles.

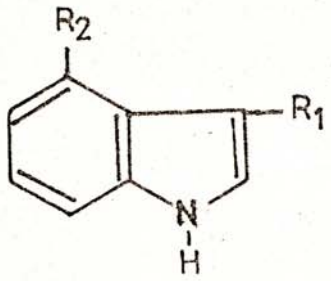
2. Chemical structure

The most important natural auxins are indole compounds. Apart from IAA several other physiologically active indole compounds have been isolated from plants. One of these is indole-3-acetonitrile, (IAN), [CXXII], first isolated by Jones et al[89] from cabbage. It has since been detected mainly by chromatographic evidence in many plants, for example tomato and grape. It is now widely believed that IAN is not active per se but owes its activity in certain biological assays to the fact that the tissues concerned can convert it to IAA enzymatically.

Some of the other naturally occurring indole compounds which may be auxins, precursors of auxins or metabolites of auxins are ethyl and methyl esters of indole-3-carboxylic, indole-3-acetic, indole-3-propionic and indole-3-butyric acids. The methyl ester of 4-chloroindole-3-acetic acid [CXXIII] has also been isolated from methanol extracts of immature pea seeds and shown to be active in auxin tests[90].

Some non-indole auxins have been isolated from plants. Examples of these are phenylacetamide and p-hydroxybenzoic acid.

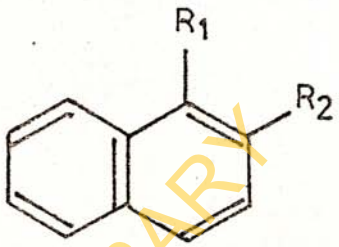
Many synthetic compounds were tested for physiological activities associated with the natural auxins. This led to the discovery of the synthetic auxins of which a large number are now known. Some of these are naphthalene-1-acetic acid [CXXIVa], naphthalene-2-acetic acid [CXXIVb], phenoxyacetic acid [CXXVa] and 2,4-dichlorophenoxyacetic acid (2,4-D) [CXXVb][91,92].



[CXXI] $R_1 = CH_2COOH$, $R_2 = H$

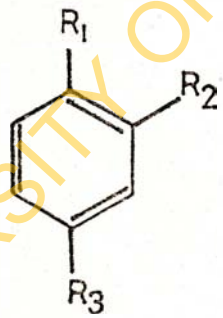
[CXXII] $R_1 = CH_2CN$, $R_2 = H$

[CXXIII] $R_1 = COOCH_3$, $R_2 = Cl$



[CXXIVa] $R_1 = CH_2COOH$, $R_2 = H$

[CXXIVb] $R_1 = H$, $R_2 = CH_2COOH$



[CXXVa] $R_1 = OCH_2COOH$, $R_2 = H$, $R_3 = H$

[CXXVb] $R_1 = OCH_2COOH$, $R_2 = Cl$, $R_3 = Cl$

Fig. 3 Some Auxins

3. Identification and characterization

The auxins just like the GAs occur in very small concentrations in plants. Their identification therefore involves the use of sensitive methods.

The extraction procedure outlined for the gibberellins can also be used for the auxins. The auxin esters and any neutral auxin will be in the neutral ethyl acetate fraction. The acidic ethyl acetate fraction will contain the free acidic auxins.

Initially the various identifications of auxins in plants were based on chromatographic evidence coupled with biological assays. Paper chromatography and thin-layer chromatography were extensively used[93,94] and tables of Rf values for several auxins in a number of solvents have been published[93].

Most of the several bioassays[95] that have been developed for detecting auxins are based on the ability of auxins to promote growth extension in plant sections. The first auxin bioassay, the 'Avena coleoptile curvature test' was developed by Went in 1928. Oat grains are grown in a suitable medium in the dark at about 25°C. When the coleoptiles are about 25mm long, the tip (about 2mm) of each coleoptile is cut off, thereby removing the main source of endogenous auxin of the coleoptile. One agar block, containing the auxin or plant extract to be tested is then placed on one side of the cut tip of each coleoptile. The seedling is then left in the dark for about two hours. The response is determined by measuring the curvature of each coleoptile.

Another bioassay also involving the use of oat or wheat coleoptiles has been developed. In this assay the lengths of cut segments of coleptiles immersed in solutions of the auxin or plant extract to be tested are measured.

Many chemical tests which give colour reactions with indole auxins have been developed[95]. These colour reactions have proved very useful in identifying auxins on paper and thin-layer chromatograms. One example of these chemical tests is the Ehrlich reaction test. In this test a mixture of p-dimethylaminobenzaldehyde and hydrochloric acid (HCl) give a range of purple and blue colours with indole compounds. The test has been used for the identification of auxins on paper and thin-layer chromatograms. For paper chromatograms, the identification can be carried out by dipping the paper in a 4:1 solution mixture of acetone and 10 per cent dimethylaminobenzaldehyde in concentrated HCl. The test can detect as low as 1 microgram of indole auxin on a chromatogram. For thin-layer chromatograms, the plate can be sprayed with an ethanolic solution of the p-dimethylaminobenzaldehyde and then kept over concentrated HCl.

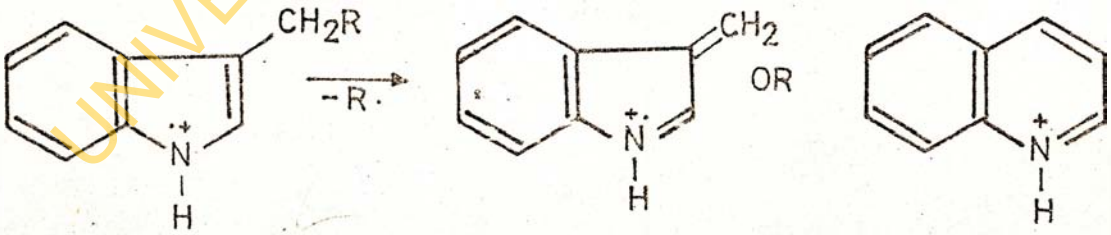
A modification of the Erlich test employs p-dimethylamino-cinnamaldehyde instead of p-dimethylaminobenzaldehyde. When this is applied as a spray in a one per cent solution in a 50:50 mixture

of ethanol and 6NHCl, it gives a blue colour with indoles.

Most of the chemical colour tests however are positive with indole compounds in general and are not specific for auxins. Some of them are not even completely specific for indole compounds.

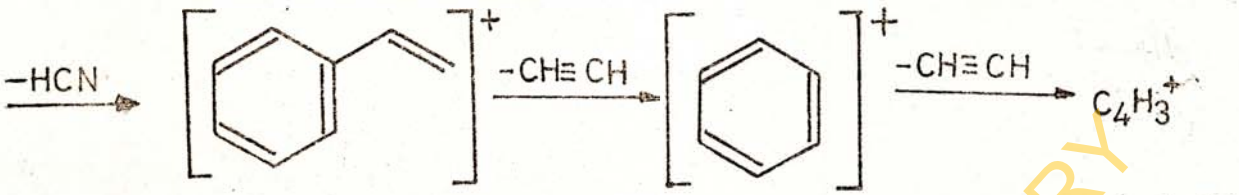
Fluorometry, gas-liquid chromatography and mass spectrometry have also been used extensively for identifying auxins[96-98]. The mass spectra of the auxin indoles normally show the molecular ion. Auxins of the type indole-3-CH_2R [CXXVIa] give a characteristic peak at m/e 130 which is usually the base peak. The structure of the ion at m/e 130 is believed[97] to be either [CXXVIb] or the quinolinium ion [CXXVII]. There is also an ion at m/e 103 [CXXVIII] due to the loss of hydrogen cyanide from the quinolinium ion. This may then lose acetylene moieties to give an ion at m/e 77 [CXXIX] and at m/e 51 [CXXX][98].

SCHEME 25



[CXXVIa] [CXXVIb] [CXXVII]

Scheme 25 contd·



m/e 103 [CXXVIII] m/e 77 [CXXIX] m/e 51 [CXXX]

4. Biosynthesis and metabolism

The investigations carried out so far on the biosynthesis of auxins have centered mainly on IAA. The amino-acid tryptophan [CXXXI] is believed to be the precursor of IAA.

Many workers have reported that labelled tryptophan (TPP) was converted to labelled IAA in many plants. For example Gibson et al[99] reported that ^{14}C-tryptophan was converted to ^{14}C-IAA in tomato and barley shoots.

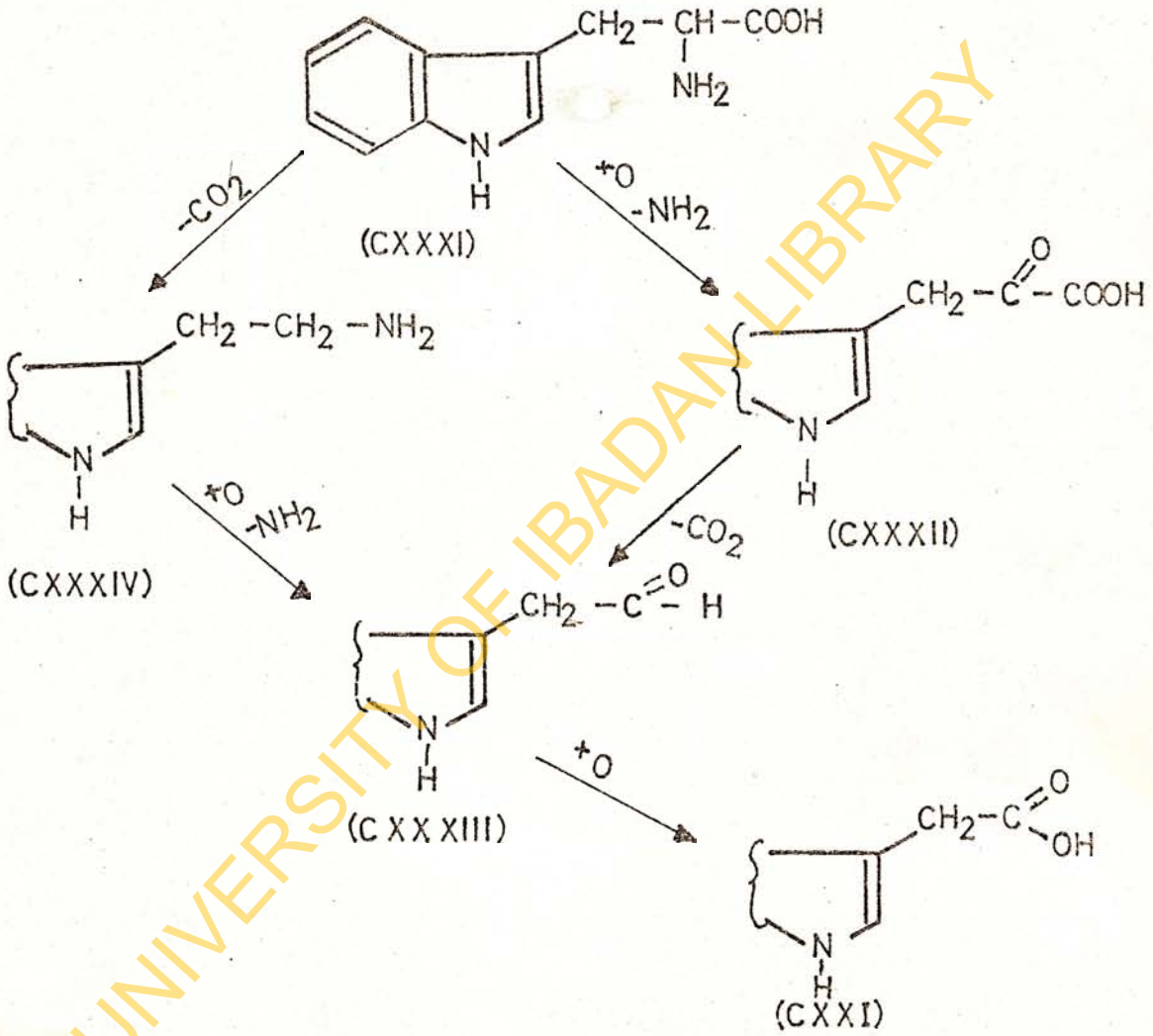
More conclusive evidence showing tryptophan as the main precursor of IAA in plants was provided by Erdmann and Schiewer[100] using double labelling technique. They fed ^{3}H-serine and ^{14}C-indole to sterile pea seedlings and non-sterile oat coleoptiles. They determined the ^{3}H/^{14}C ratios of the tryptophan and the IAA that

were produced from the labelled serine and indole. They then supplied $^{3}H,^{14}C$-tryptophan to the seedlings and coleoptiles instead of the labelled serine and indole. They determined the $^{3}H/^{14}C$ ratio of the IAA obtained. They found that the relative labelling ratio $^{3}H/^{14}C$-tryptophan/$^{3}H/^{14}C$-IAA was the same in each case. This showed that the conversion of indole to IAA passed through tryptophan without any significant bypass.

It is generally believed that the main pathway from tryptophan to IAA in most plants is through indole-3-pyruvic acid [CXXXII] and indole-3-acetaldehyde [CXXXIII]. The first intermediate, indole-3-pyruvic acid (IPyA) has not been isolated from plants but its presence has been demonstrated in many plants.

Gibson et al[99] demonstrated the in vivo conversion of labelled tryptophan to labelled IPyA in barley and tomato shoots. They fed labelled tryptophan to the shoots and then added unlabelled carrier IPyA to the resulting metabolites that were extracted from the shoots. The 2,4-dinitrophenylhydrazone of the IPyA was prepared to stabilise it and it was found to be radioactive after separation on thin-layer chromatogram. The in vivo conversion of IPyA to indole-3-acetaldehyde (IAAld) has not been demonstrated but many plants produce IAA when fed with IAAld.

SCHEME 26

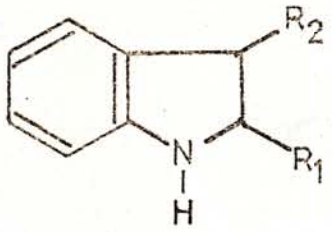


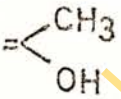
Another biosynthetic pathway from tryptophan to IAA which is believed to occur in some plants is the tryptamine [CXXXIV] pathway. In this pathway the tryptophan is converted to IAAld through tryptamine (TNH_2) [CXXXIV] instead of through IPyA as in the first pathway. TNH_2 has been found to be endogenous in several plants for example barley and tomato shoots[101]. It has however not been detected in many others like pea and bean[101].

Gibson et al[99] reported that tomato and barley shoots converted labelled tryptophan to labelled TNH_2 and also converted labelled TNH_2 to labelled IAA. They demonstrated the participation of IAAld in this pathway by adding carrier unlabelled IAAld to the metabolites obtained after feeding ^{14}C-tryptophan to the shoots. They prepared the 2,4-dinitrophenylhydrazone derivative of the IAAld in each case and found that both hydrazones were radioactive.

The sites of biosynthesis of auxins are believed to be apical meristems and other actively meristematic organs, for example immature leaves and flowers.

IAA is metabolised to several products[85,102]. Some of these are 3-hydroxy-3-methyl-2-oxindole [CXXXV], 3-hydroxymethyl-2-oxindole [CXXVI], and 3-methylene-2-oxindole [CXXXVII].



[CXXXV] $R_1 =$ =O, $R_2 =$ 

[CXXXVI] $R_1 =$ =O, $R_2 = CH_2OH$

[CXXXVII] $R_1 =$ =O, $R_2 = CH_2$

Fig. 4 Some metabolites of IAA

5. Uses

Some auxins are used commercially in agriculture[103]. They are used to induce flowering in some fruits for example pinapple. They are also used to induce fruit-set in some others such as tomato where the application of auxin can replace the need for the pollination of the flower. They are used to control fruit drop, for example to prevent preharvest drop in apples. Some of the auxins are also used as herbicides e.g. 2,4-D [CXXVb].

The Cytokinins

The cytokinins are a group of plant hormones which promote cell division[104]. The various investigations leading to the isolation of the cytokinins followed from the observation that certain plant extracts and fluids promote the growth of 'tissue cultures' mainly by cell division. Further investigations led to the detection of a cell division factor in deoxyribonucleic acid (DNA).

A new cell division factor called kinetin was isolated from DNA by Miller et al[105]. The extraction procedure used involved stirring the DNA in water and autoclaving before extraction with organic solvent. Kinetin was identified as 6-furfurylaminopurine [CXXXVIII][105] and it is now generally believed to be an artefact probably produced by chemical degradation of the DNA molecule during the autoclaving.

Many physiologically active compounds, structurally related to kinetin have since been isolated from plants and others have been synthesized. They are all referred to as cytokinins.

1. Physiological roles

The cytokinins perform various physiological functions in plants[106]. These include:

(a) promotion of cell division in tissue cultures and also in intact plants,

(b) delay of senescence in plant organs probably by inhibiting protein breakdown or promoting protein synthesis,

(c) mobilization of assimilates,

(d) enhancement of the resistance of plants to adverse conditions,

(e) promotion of bud development in a variety of plants and tissues, and

(f) regulation of the activities of some enzymes.

2. Chemical structure

The presence of an adenine molecule with the purine ring intact and with a N^6 -substituent of moderate size appears to be one of the principal structural prerequisites for a high level of cytokinin activity. There are exceptions however, for example diphenylurea and its derivatives are active. The naturally occurring cytokinins are mainly adenine derivatives.

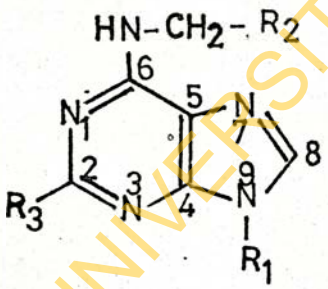
The first naturally occurring cytokinin to be isolated was zeatin [CXXXIX]. It was isolated from methanol extract of immature maize kernels[107]. The extract was purified with ion-exchange column chromatography and paper chromatography.

The structure of zeatin was determined by Letham et al[108] by a combination of chemical and spectrometric methods. When oxidised with nitric acid or manganese dioxide it yielded adenine. On chromatograms, zeatin gave indications that it was a purine. The pKa values for zeatin were 4.4 and 9.8 (in water) indicating that positions 1,3,7 and 9 of the adenine molecule were unsubstituted. Substitution was therefore believed to be at the 6-amino-group. Comparison of the ultraviolet and mass spectra of zeatin with those of 6-(substituted amino) purines indicated that the amino-group was monosubstituted. The n.m.r. spectrum of the picrate determined in deuteropyridine/deuterium oxide had two one-proton singlets at δ7.88 and δ7.96 assigned to the protons at positions 2 and 8 of adenine. The basic structure of the zeatin molecule was thus believed to be a purine molecule substituted at N^6-position.

The structure of the substituent group was deduced from the mass and n.m.r. spectra of zeatin. The mass spectrum showed a molecular ion at m/e 219, and a base peak at m/e 202 due to the loss of a hydroxyl group. There was a prominent peak at m/e 188 due to the loss of CH_2OH from the molecular ion. Prominent ions at m/e 148 m/e 136 and m/e 135 which are characteristic[109] of adenine derivatives were also obtained.

The n.m.r. spectrum showed eight protons in addition to the two already detected in the purine molecule. There was a peak at δ1.53 (3H, doublet) assigned to a methyl group adjacent to a double bond. A 4H, multiplet at around δ3.84 was assigned to two methylene groups both adjacent to either oxygen or nitrogen. A peak at δ5.42 (1H, multiplet) was assigned to an olefinic proton.

The structure of zeatin was thus deduced to be 6-(4-hydroxy-3-methyl-trans-2-butenylamino) purine [CXXXIX]. This structure was confirmed by synthesis[110]. The condensation of the amino-alcohol [CXL] with 6-methylmercaptopurine [CXLI] in a sealed tube at 134° gave a product which on purification was identical with zeatin.



[CXXXVIII] $R_1 = H$, R_2 =  , $R_3 = H$

[CXXXIX] $R_1 = H$, $R_2 = -CH=C(CH_2OH)(CH_3)$, $R_3 = H$

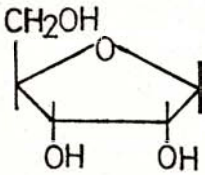
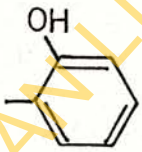
Fig.5. Some Cytokinins

(CXLII) $R_1 = R_3 = H$, $R_2 = -CH_2-CH(CH_2OH)(CH_3)$

(CXLIII) $R_1 = R_3 = H$, $R_2 = -CH=C(CH_3)(CH_3)$

(CXLIV) $R_1 = H$, $R_2 = -CH=C(CH_2OH)(CH_3)$, $R_3 = CH_2S$

(CXLV) $R_1 = H$, $R_2 = -CH=C(CH_3)(CH_3)$, $R_3 = CH_3S$

(CXLVI) R_1 =  , R_2 =  , $R_3 = H$

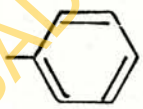
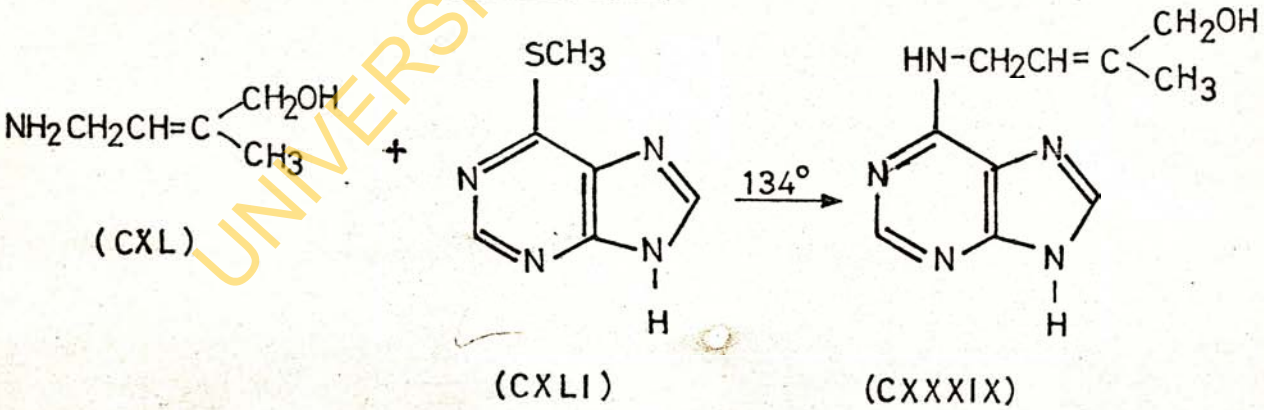
(CXLVII) $R_1 = R_3 = H$, R_2 = 

Fig. 5 contd. Some Cytokinins

SCHEME 27



(CXL) (CXLI) (CXXXIX)

Another cytokinin, named dihydrozeatin [CXLII] was isolated from immature seeds of Lupinus luteus by Koshimizu et al[111]. The methanol extract of the seeds was purified by column and paper chromatography and an active compound was obtained. This compound had the same Rf with zeatin on paper chromatograms in three different kinds of solvent systems. The infrared spectrum of its picrate was however different from zeatin picrate.

The n.m.r. spectrum of the picrate in deuteropyridine contained the following peaks: A 3H-doublet at δ1.15 assigned to a methyl group adjacent to a secondary carbon atom (H-C-CH_3). A 3H multiplet at δ1.60-2.40. A 2H-doublet, at δ3.78 (H-C-CH_2OH), a 2H-multiplet at δ4.13 (>N-CH_2-), and a 2H-singlet at δ8.98 (protons at positions 3 and 5 of picric acid). Also observed were two one-proton singlets at δ8.47 and 8.78 assigned to the protons at position 2 and 8 of adenine.

The u.v. absorption spectra $\lambda_{max}^{0.1N\ NaOH}$ 275 nm and 282 nm (shoulder), λ_{max}^{EtOH} 269 nm, and $\lambda_{max}^{0.1N\ HCl}$ 273 nm indicated a N^6(alkyl-substituted) adenine.

The structure was deduced to be 6-(4-hydroxy-3-methyl butylamino) purine [CXLII]. This structure was confirmed by synthesis. Catalytic hydrogenation of zeatin gave dihydrozeatin as one of the products and this was identical with the natural material.

A cytokinin was isolated[112] from liquid cultures of a bacterium, Corynebacterium fascians, (a plant pathogen which induces multiple bud formation in certain trees). The structure was elucidated by Helgeson and Leonard[113] to be 6-(3-methyl-2-butenylamino) purine (isopentenyl adenine, IPA) [CXLIII].

The u.v. spectrum indicated a N^6(alkyl-substituted) adenine and the n.m.r. spectrum showed the presence of two methyl group instead of the one methyl group in zeatin. The mass spectrum showed the general pattern for purine derivatives, similar to that of zeatin. This cytokinin has since been identified in some higher plants for example in immature peas.

The riboside of IPA was isolated[114] as one of the constituent nucleosides of soluble RNA of yeast. Soluble ribonucleic acid extracted from yeast was hydrolysed enzymatically to its constituent nucleosides. The mixture was separated by column chromatography. IPA-riboside was obtained and crystallized.

The n.m.r. spectrum (in CD_3COCD_3-D_2O) exhibited the basic pattern associated with adenosine and also contained the following peaks. A split peak at δ1.75 (6H-two vinyl methyl groups), a one-proton multiplet at δ5.4 (vinyl proton) and a three-proton multiplet at δ4.2 assigned to the methylene group attached to N^6 and to the C-4 proton of ribose.

The mass spectrum had a molecular ion at m/e 335 and strong peaks at 292 and 203 (free base). There were also prominent peaks at m/e 88, 160, 140, 136 and 135 (adenine).

Some other cytokinins which have been identified[115] as constituent nucleosides of transfer RNA after hydrolysis are ribosides of CH_3S-zeatin [CXLIV], and CH_3S-IPA [CXLV]. Transfer RNA extracted from wheat was hydrolysed enzymatically and the resulting ribonucleosides were extracted with ethylacetate and separated on Sephadex LH-20 columns and on paper. Their u.v. spectra were indicative of N^6-(alkyl substituted) adenine.

The mass spectra of CH_3-S-zeatin and CH_3-IPA ribosides had a fragmentation pattern similar to those of zeatin and IPA ribosides respectively except that the molecular weights were 46 atomic mass units higher in each case. Their structures were deduced to be 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosyl purine, (CH_3S-zeatin riboside) [CXLIV] and 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine, (CH_3S-IPA riboside) [CXLV].

Another cytokinin, 6-(O-hydroxybenzylamino) purine was isolated[116] from leaves of Populus robusta as its ribofuranoside [CXLVI]. The u.v. spectrum of the compound $\lambda_{max}^{(EtOH\ neutral)}$ 266nm, $\lambda_{max}^{(EtOH\ pH_2)}$ 260 nm, $\lambda_{max}^{(EtOH\ pH\ II)}$ 265 nm, indicated that it was a N^6-substituted adenosine.

The mass spectrum of the compound which had principal peaks at m/e 373, 284, 270, 241, 224, 178, 164, 148, 135 (base peak), 121, 120, 119, 108, 106, 78, 66 as well as a molecular ion at m/e 661 suggested that the compound was a N^6(hydroxybenzyl) adenosine. This was confirmed by high resolution mass spectrometry.

The position of the hydroxyl group on the benzene ring was determined by comparing the mass spectra of the three synthetic isomers with that of the natural product. It was then confirmed by co-chromatography on a GLC system which clearly separated the three isomers.

The syntheses of the three isomers were effected by condensing 6-chloropurine-9-β-D-riboside with the appropriate hydroxybenzylamine in refluxing methanol.

Many biologically active cytokinins have been synthesized. The most active of these are adenine derivatives for example 6-benzylaminopurine [CXLVII].

The two methods that are usually used for the syntheses of cytokinins are: (a) displacement of alkylthio group[117] or chlorine[118] at the 6-position of the purine ring and (b) direct alkylation[119]. The displacement reaction has been used to synthesize N^6-substituted adenines and adenosines. For example, kinetin [CXXXVIII] was synthesized by reacting 6-methylmercaptopurine [CXLI] with

furfurylamine at 115-120° for 9 hrs.[105] 6-(o-hydroxybenzylamino) purine riboside [CXLVI] was synthesized by reacting 6-chloropurine riboside with o-hydroxybenzylamine in refluxing n-butanol. Thus the reaction basically involves the reaction of a 6-thioalkyl purine or a 6-chloropurine with an appropriate amine.

The second method of direct alkylation is usually used for synthesizing cytokinin ribosides and ribotides. The reaction is based on the fact that a substituent at the 9-position of the purine ring directs alkylation to the 1-position. Therefore 1-substituted adenines are readily prepared by reacting adenine (substituted in the 9-position) with an appropriate alkyl halide. The 1-substituted adenines are then readily rearranged to N^6-substituted adenines by heating in alkali[119].

3. Isolation and Identification

The extraction procedure usually used for the cytokinins involves the grinding of the plant material in aqueous methanol. The methanol is then removed under reduced pressure. The pH of the resulting aqueous phase is adjusted to about 3.0 and the aqueous phase is extracted with ethyl acetate to remove acidic impurities. The pH of the aqueous phase is adjusted to 7.0 and it is extracted with water-saturated n-butanol. The cytokinins will be in this n-butanol fraction. The n-butanol is then removed in vacuo.

Further purification of the extract is usually carried out by ion-exchange column chromatography and gel-filtration chromatography. Paper chromatography is also usually used for further separation. Gas chromatography is used for the separation of trimethylsilyl derivatives of the cytokinins[120]. It is also used for identification when authentic samples are available.

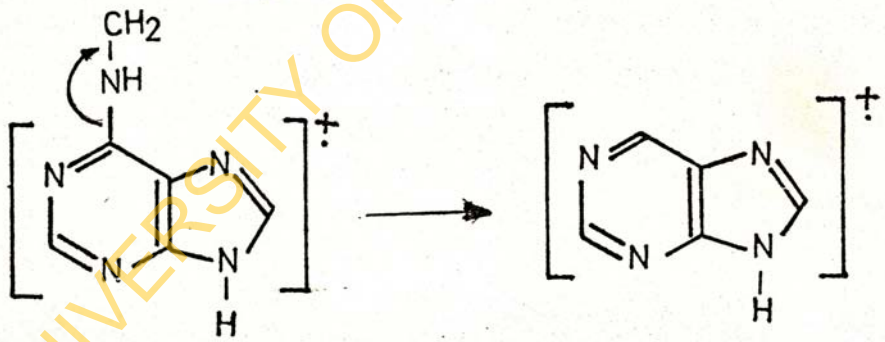
The shape and general appearance of the ultraviolet spectra of the cytokinins (adenine derivatives) under different pH conditions are used to determine the position of the substituent on adenine[121]. For example 9-substituted adenines usually have u.v. spectra which are insensitive to pH. The adenines usually absorb u.v. light strongly in the region 250nm to 280nm.

The pka values of the adenine derivatives are also used to determine the position of substitution on the adenine molecule[122]. The presence of pKa in the region below 6 indicates that positions 1, 3 and 7 are not substituted. If the pKa lies in the region 8 - 11, it shows that the N-H group is still present in the five-membered ring of adenine. If however there is no pKa value in the region 8 - 11 it means that there is no ionizable hydrogen in the five-membered ring indicating that position 9 of adenine is substituted.

Mass spectrometry has been employed extensively for the elucidation of the structures of the various cytokinins[109]. The 6-alkylaminopurinyl structures of most of the cytokinins show some characteristic ions in their mass spectra. Some of these are ions at m/e 148 [CXLVII], 136 [CXLVIII], 135 (adenine ion) [CXLIX], 119 (purine ion) [CL], and m/e 108 (adenine - HCN) [CLI] which may be derived as shown in Scheme 28.

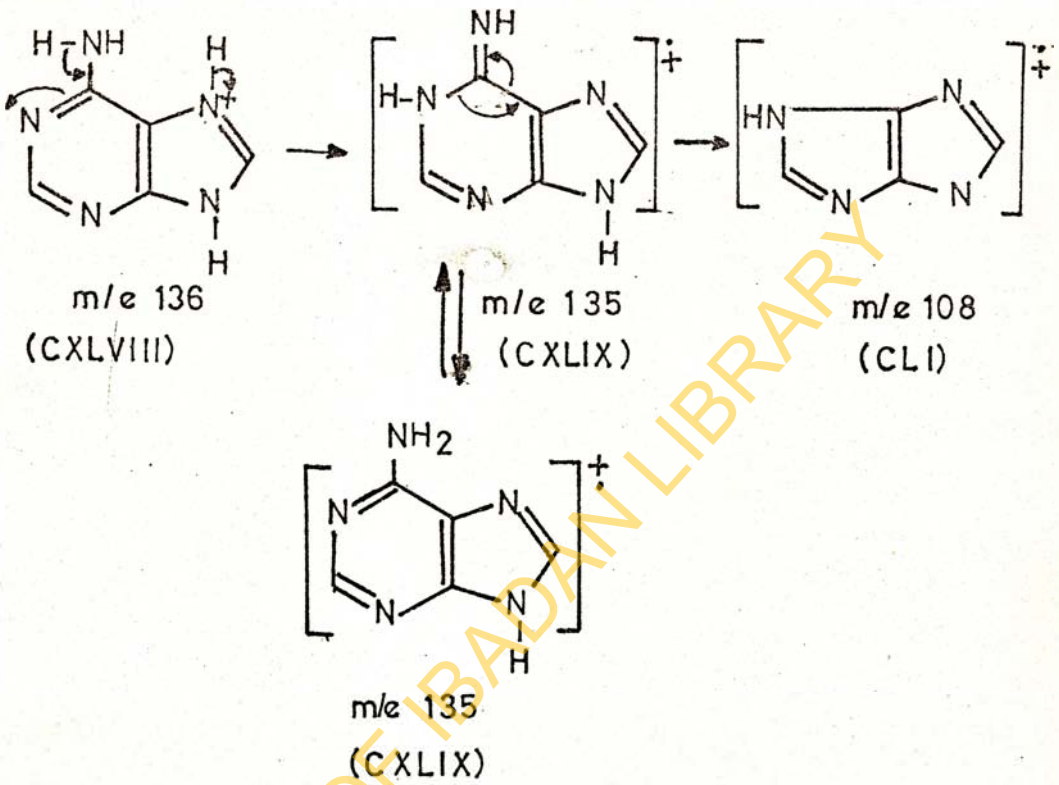
Combined gas-chromatography-mass spectrometry has also been used for the identification of trimethylsilyl derivatives of cytokinins[123].

SCHEME 28



m/e 148 (CXLVII) m/e 119 (CL)

Scheme 28 contd·



Some bioassays have also been developed for the detection of cytokinins[124,125]. Some of these are based on the ability of the cytokinins to stimulate cell division in tissue cultures (e.g. tobacco callus assay). Some others are based on the ability of cytokinins to delay senescence and loss of chloroplasts in detached leaves, and to induce the formation of buds in some plants. The bioassays based on cell-division are usually the most reliable because of their sensitivity and specificity.

4. Biosynthesis and metabolism

The biosynthetic pathway leading to the cytokinins is still not well understood but the isoprenoid side chain is believed to be from mevalonate.

Seeds, immature fruits, roots, and root exudate have been identified as rich sources of cytokinins in higher plants.

The cytokinins are rapidly metabolised in plant tissues. Some of the metabolites of zeatin that have been isolated are 7-glucosylzeatin, 9-glucosylzeatin, zeatin riboside and zeatin riboside 5'-monophosphate[125].

The synthetic cytokinins are similarly metabolised to the glycosides and the glycotides and are in part degraded to adenine.

Abscisic acid

Abscisic acid (ABA) [CLII], a monocarboxylic, monocyclic sesquiterpenoid was first isolated as an abscission accelerating substance in cotton plants[127]. It was also isolated from sycamore leaves and yellow lupin where it caused the abscission of immature fruitlets. Since then ABA has been isolated from several plants and detected in very many others[128].

1. Physiological roles

There have been many reports[128,129] of the physiological roles of ABA in plants. Some of these functions are:

(a) abscission acceleration,

(b) induction of dormancy,

(c) regulation of stomatal opening,

(d) involvement in some aspects of ripening process, and

(e) inhibition of the synthesis of some enzymes, for example α-amylase in cereal aleurone.

2. Chemical structure

Ohkuma et al[130] proposed a structure for ABA based on spectral data. The molecular formula was deduced to be $C_{15}H_{20}O_4$ from chemical analysis and the mass spectrum of ABA which showed a molecular ion at 264 a.m.u.

The infrared spectrum of ABA showed hydroxyl group (3405 cm^{-1}), carboxylic acid group (2300 cm^{-1}) and a conjugated keto group (1650 cm^{-1}). The presence of three bands at 1674 cm^{-1}, 1623 cm^{-1}, and 1600 cm^{-1} was believed to indicate the presence in ABA of a group similar to sorbic acid (2,4-Hexanedienoic acid). A strong peak at 978 cm^{-1} indicated the presence of a trans substituted double bond.

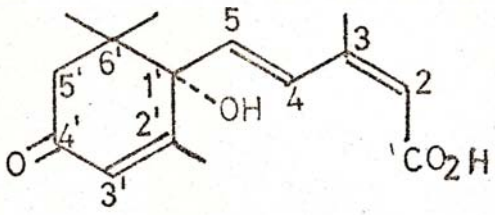
The n.m.r. spectrum showed the presence of four methyl groups. Two of these were on saturated carbon (δ1.10 and 1.17) and two were vinyl methyl groups (δ1.99 and 2.10). Two strong peaks at δ2.41 and δ2.47 were believed to represent a methylene group adjacent to a carbonyl group. There were four vinyl protons at δ5.79, 5.98, 6.17 and 7.81.

The mass spectrum had prominent M^+-56 (due to the loss of isobutylene from the ring) and M^+-111 due to the loss of the side chain.

On the basis of the spectal data they deduced the structure of ABA to be 3-methyl-5-(1'-hydroxy-4'-oxo-2',6',6'-trimethyl-2'-cyclohexen-1-yl)-cis, trans-2,4-pentadienoic acid [CLII]. This structure was confirmed by synthesis[131].

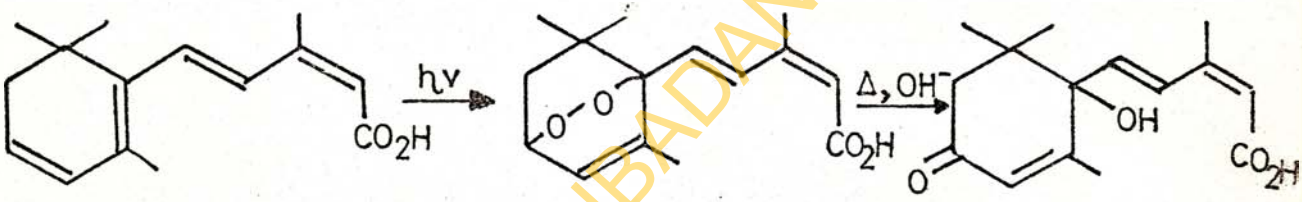
The synthesis consisted of the photo-sensitized epidioxidation of 3-methyl-5-(2',6',6'-trimethylcyclohexa-1',3'-dienyl)-cis,trans-2,4-pentadienoic acid [CLIII] to give an epidioxide [CLIV]. This was rearranged by heating at 100°C in aqueous sodium hydroxide giving racemic ABA [CLV] after acidification.

The stereochemistry of the tertiary hydroxyl group of natural ABA is α and the absolute configuration of natural ABA is (S).[128] It is strongly dextro-rotatory ([α]D + 430°).



(S)-(+)-ABA (CLII). R = H

SCHEME 29

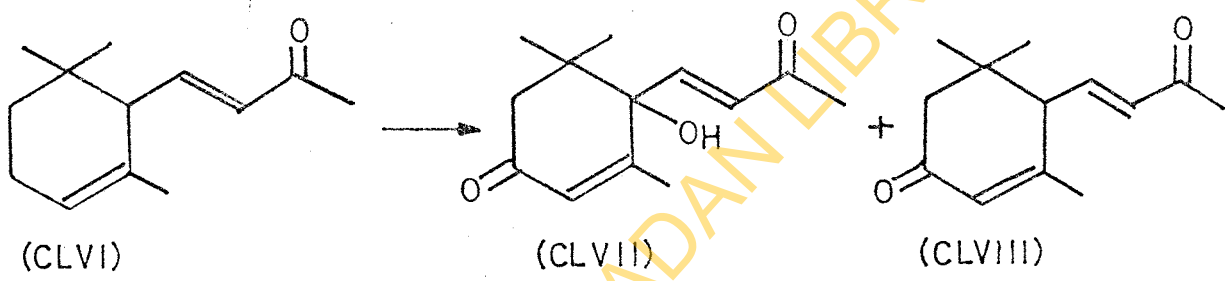


(CLIII) (CLIV) (±)ABA (CLV)

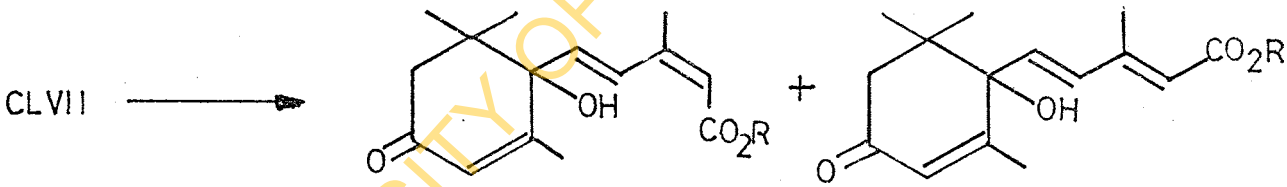
An improved synthesis of ABA was reported by Roberts et al[132] starting with α-ionone [CLVI]. α-Ionone in t-butyl alcohol was oxidised with t-butyl chromate to give 1-hydroxy-4-keto-α-ionone [CLVII] and 4-keto-$^{\alpha}$-ionone [CLVIII] in a 4:1 ratio approximately. The side chain was then extended by Wittig reaction using triphenyl carbethoxymethylene phosphorane. Thus refluxing the 1-hydroxy-4-keto-α-ionone in toluene with the phosphorane $[(C_6H_5)_3P{=}CHCOOC_2H_5]$ gave a 1:1 mixture of racemic ABA ethyl ester [CLIX] and the

2-trans isomer [CLX]. The mixture on hydrolysis with base gave racemic ABA [CLV] and the 2-trans isomer [CLXI] in a 1:1 ratio and these were separated on t.l.c.

SCHEME 30



(CLVI) (CLVII) (CLVIII)



(CLIX) R = $-CH_2CH_3$ (CLX) R = $-CH_2CH_3$

(CLV) R = H (CLXI) R = H

3. Isolation and identification

The extraction procedure used for the gibberellins and auxins can also be used for abscisic acid. Free ABA will be extracted in the acidic ethyl acetate (AE) fraction. Any ABA ester present will be in the neutral ethyl acetate (NE) fraction

and ABA glucoside in the acidic n-butanol (AB) fraction. Further purification of the extract can be by the various chromatographic methods.

Several methods have been developed for identifying ABA in purified extracts. One of these is the spectropolarimetric method[133,134] which utilises the unique optical rotatory dispersion (ORD) of ABA. This has an intense positive 'cotton effect' with extrema at 289nm and 246nm. The ORD spectrum is affected by pH and so it is usually measured in 0.005N H_2SO_4 or the spectrum of the methyl ester which is unaffected by pH is measured. The 'cotton effect' of the ORD is so large that if the extract is well purified, interference from other substances may be negligible. This ORD method can therefore be used to determine ABA present in an extract both qualitatively and quantitatively, provided there is no other optically active substance in the extract.

The ultraviolet spectrum of ABA can also be used to identify it in highly purified extracts. The u.v. spectrum[134] is also affected by pH. In acidic conditions the absorption maximum occurs at 262nm (ε = 21,400) with a shoulder at 240nm. In basic conditions the maximum occurs at 245nm and it is about 20% more intense.

Milborrow[134] developed a method for the determination of concentrations of ABA in plant tissues. In this method called 'racemate dilution' (RD) method, a known amount of synthetic, racemic

ABA is added to the crude plant extract. The extract is purified and the total amount of ABA remaining after the purification is determined from the u.v. absorption. The (S)-(+)-ABA remaining after the purification is determined by the ORD method. From these determinations, the amount of natural, ((S)-(+)) ABA originally present in the plant tissue can be calculated. The main disadvantage of the use of u.v. and ORD for identifying and estimating ABA is the very high degree of purification that is usually necessary.

Thin-layer chromatography has been used for identifying ABA. It can be separated from its 2-trans isomer by this method.

GLC is also very useful for identifying ABA in plant extracts, and rigorous purification may not be necessary[135]. Methyl and trimethylsilyl esters of ABA can readily be separated on GLC and identified by comparison with authentic samples.

The use of GC-MS makes the identification of ABA in extracts possible without the availability of authentic samples.

The mass spectrum of methyl ester of ABA shows a molecular ion at m/e 278 and prominent peaks at m/e 260 ($M^+ - H_2O$), 246 ($M^+ - CH_3OH$), 222 (M^+-isobutylene), 190, (base peak), ($M^+ - CH_3OH$ and isobutylene), 162, 134 and 125.

Two chemical colour tests have been developed for ABA. The first test, reported by Antoszewski and Rudnicki[136] is based on the fact that ABA gives a yellow-green fluorescent colour after heating with sulphuric acid on silica gel. This is used to detect ABA on silica gel thin-layer chromatograms.

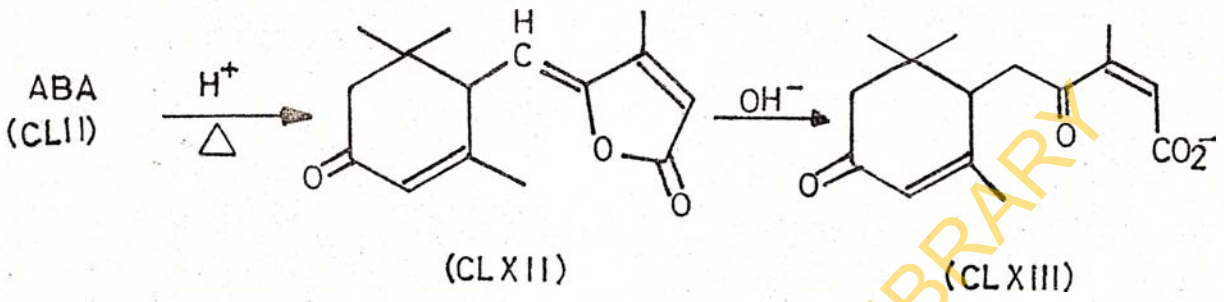
The other colour test was first reported by Mallaby and Ryback[137] and is based on the fact that ABA gives a lactone [CLXII] as the major product when heated with acid. This lactone gives an intense characteristic transistory violet-red colour with alkali. This is used as a sensitive qualitative test for ABA.

Several bioassay systems[129] have been used to detect ABA. They include: (a) acceleration of abscission in excised abscission zones (explants), (b) inhibition of coleoptile curvature or straight growth, (c) inhibition of seed germination, (d) the inhibition of growth of rice seedlings and (e) stomatal closure assay.

4. Chemistry

Much work has not been done on the chemistry of ABA. Mallaby and Ryback[137] reported that when ABA was heated with a mixture of formic acid and concentrated hydrochloric acid, a lactone [CLXII] was obtained as the major product. The lactone gave an intense violet-red colour with alkali. The lactone was believed to be hydrolysed to the keto acid salt [CLXIII] by alkali.

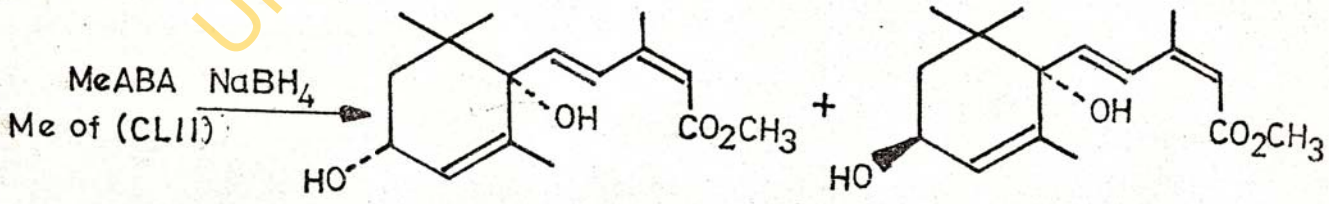
SCHEME 31



(CLXII) (CLXIII)

ABA methyl ester is reduced in aqueous methanolic sodium borohydride to give approximately equal mixtures of the 1',4'-cis-diol [CLXIV] and the 1',4'-trans-diol [CLXV][138]. These diols are readily reoxidized to ABA by manganese dioxide in dry MeOH.

SCHEME 32



(CLXIV) (CLXV)

ABA exchanges six of its carbon-borne hydrogen atoms with water at pH 11 and above. These are the C-2'-methyl hydrogens, the C-3' hydrogen and the C-5' methylene hydrogens. This has been used as a basis for preparing deuterated ABA[139].

5. Biosynthesis

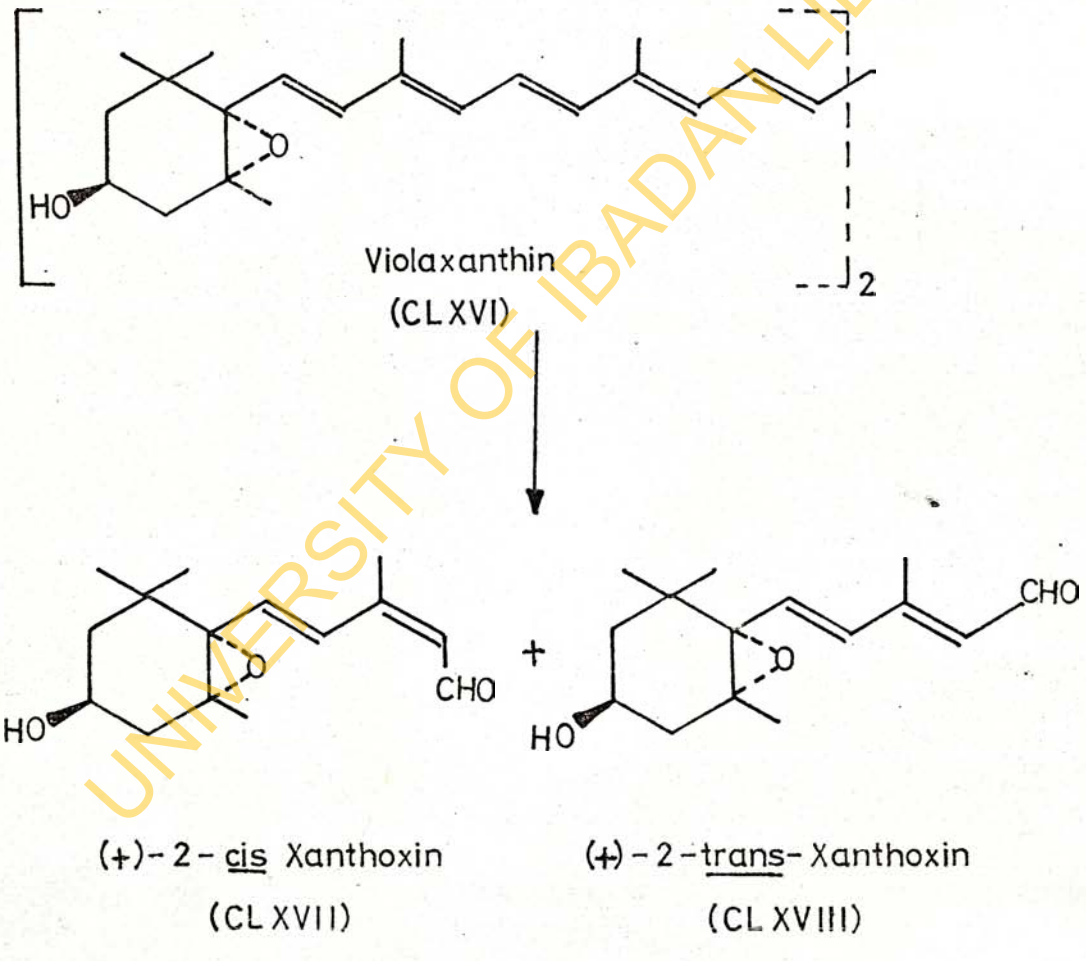
The terpenoid nature of ABA was demonstrated by the fact that many plants converted labelled mevalonate into ABA[138,140]. This was first demonstrated by Noddle and Robinson[140] who reported that [^{14}C] mevalonic acid was incorporated into ABA by avocado and tomato fruits. They supplied labelled mevalonic acid to ripening fruits and extracted the ABA two days later. The ABA contained the label from the mevalonic acid.

The pathway from mevalonate to ABA is still not very certain. Two pathways have been postulated. In the first pathway known as the "carotenoid pathway", it is postulated that ABA is derived from a carotenoid, especially violaxanthin [CLXVI][141]. In the second pathway called the "direct synthesis pathway", it is postulated that a C_{15} precursor is cyclized and then converted into ABA[142].

In order to test the 'carotenoid pathway', Taylor and Smith exposed some carotenoids on damp filter paper to bright light. They found that violaxanthin gave rise to a strong inhibitor which was later characterized[143] to be approximately equal amounts of

2-cis-xanthoxin [CLXVII] and 2-trans-xanthoxin [CLXVIII]. The 2-cis isomer was found to be the active component. Xanthoxin has since been detected in extracts of many plants, for example dwarf bean and wheat seedlings[144].

SCHEME 33



(+)-2-cis Xanthoxin (CLXVII)

(+)-2-trans-Xanthoxin (CLXVIII)

Taylor and Burden have shown that labelled 2-cis-xanthoxin is converted to ABA by shoots of tomato and bean[145]. They fed 2-[^{14}C]-cis-xanthoxin to cut shoots of tomato and dwarf bean and found that it was converted within 8 hours to ABA in yields of 10.8% and 7.0% respectively. This result and the fact that xanthoxin had been shown to be endogenous in many plants[144] were taken to suggest that carotenoids and xanthoxin may be precursors in the biosynthesis of ABA. The results did not however establish the importance of the 'carotenoid pathway' in normal biosynthesis of ABA.

On the other hand, strong evidence has been produced in favour of the 'direct synthesis' pathway[128]. One of the experiments suggesting that ABA is derived from a C_{15} precursor and not from a carotenoid, was carried out by Robinson[146]. He supplied [^{14}C]-labelled phytoene (an uncyclized carotenoid) to avocado fruit together with [^{3}H] mevalonate. He then extracted and isolated the ABA and carotenoids in the fruit after some hours. The [^{3}H] label was incorporated into the carotenoids and the ABA. The [^{14}C] from the phytoene was incorporated into carotenoids but it was not incorporated into ABA. This experiment thus showed that ABA was formed from mevalonate in avocado fruit via a route not involving a carotenoid. This strong evidence in support of the direct synthesis pathway did not however exclude the possibility that under certain circumstances the carotenoid pathway might also operate.

6. Metabolism

Several metabolites of ABA have now been isolated. It was reported[147] that when labelled ABA was supplied to tomato shoots, it was rapidly metabolised into two compounds. The first compound was identified as the glucose ester of ABA.

The second compound called 'metabolite C' [CLXIX] was more polar than ABA. The compound showed a strong positive ORD curve similar to that of ABA but displaced slightly towards longer wavelengths ((+)-extremum = 298nm, (-)-extremum = 254nm).

The ultraviolet and infrared spectra of 'metabolite C' were almost identical with those of ABA. The infrared spectrum of ' 'metabolite C' contained absorption bands at 1585, 1615 and 1666cm^{-1} indicating that the double bonds in the ring and side chain of ABA were still present in 'metabolite C'. The absorption band at 3400cm^{-1} was more intense in 'metabolite C' than in ABA. 'Metabolite C' was acetylated with acetic anhydride in pyridine, but the tertiary hydroxyl group of ABA could not have been acetylated in that way. 'Metabolite C' was therefore deduced to be a hydroxylated derivative of ABA.

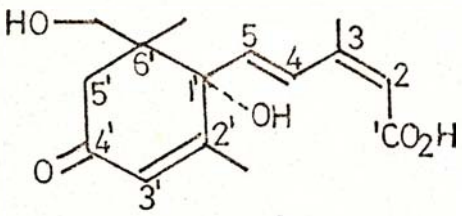
'Metabolite C' was reported[147] to be unstable, rearranging easily to give another compound, phaseic acid (PA) [CLXX]. Phaseic acid had a plain negative ORD curve between 250 and 350nm.

In the infrared spectrum of phaseic acid, the OH absorption was less intense than that of 'metabolite C'. The band at $1666cm^{-1}$ in the infrared spectrum of 'metabolite C' was absent in the spectrum of phaseic acid but there was a new band at $1715cm^{-1}$. These changes were believed to be due to the saturation of the double bond α, β to the 4'-keto group of 'metabolite C'.

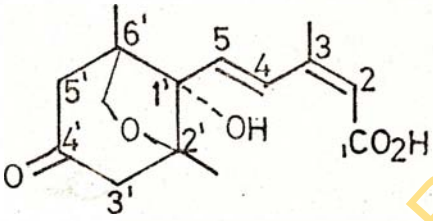
The n.m.r. spectrum of methyl phaseate showed that the 3-methylpentadienoic acid side-chain of ABA was still present in phaseic acid. The n.m.r. spectrum also contained a new methylene signal and showed the loss of the peak assigned to one of the 6'-gem-dimethyl groups present in ABA. This thus indicated that 'metabolite C' was formed by the hydroxylation of one of the C-6' dimethyl groups of ABA.

The structure of 'metabolite C' was thus deduced to be 6'-hydroxymethyl abscisic acid [CLXIX]. Subsequent attempts to isolate 'metabolite C' have been unsuccessful.

Phaseic acid was believed to be formed from 'metabolite C' by the nucleophilic attack of the hydroxymethyl on C-2', thus giving the structure of phaseic acid as [CLXX]. Phaseic acid was first isolated[148] from bean seeds (Phaseolus multiflorus).



(CLXIX)
'metabolite ' C'



(CLXX)
phaseic acid

Tinelli et al[149] reported that two compounds designated M-1 and M-2 were isolated as metabolites of ABA in excised axes of Phaseolus vulgaris. They fed 2-^{14}C-ABA to the axes and isolated the two radiactive metabolites after 48 hours.

M-2 was methylated with diazomethane and the methyl ester on oxidation with CrO_3 in acetic acid gave a compound which was identical with M-1 methyl ester. The mass spectrum of the compound (M^+ 294) was identical with that of methyl phaseate.

M-2 was acetylated with acetic anhydride in pyridine at room temperature but M-1 did not react with acetic anhydride in this way. M-2 did not react with 2,4-dinitrophenylhydrazine but M-1 did. M-1 methyl ester gave two main products when reduced with sodium borohydride and one of these co-chromatographed with M-2 methyl ester.

From the above data M-1 was deduced to be phaseic acid and M-2,4'-dihydrophaseic acid (DPA) [CLXXI].

The mass spectrum of the methyl ester of 4'-dihydrophaseic acid showed a molecular ion at m/e 296 and a base peak at m/e 43.

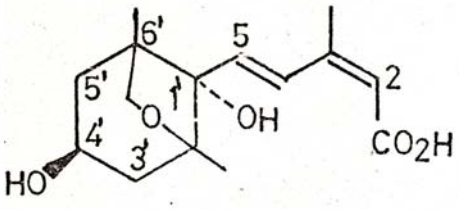
Naturally occurring 4'-dihydrophaseic acid (DPA) was first isolated from methanol extract of mature bean seeds by Walton et al[150].

The epimer of DPA, referred to as epi-4'-dihydrophaseic acid, (epi-DPA), [CLXXII] was identified as a minor metabolite of ABA in Phaseolus vulgaris[151]. Racemic ABA (labelled) was fed to bean shoots. After extraction and purification of the acidic fraction by thin-layer chromatography on silica gel, (+)-ABA was found to have been metabolised to phaseic acid, DPA and epi-DPA. The PA was methylated and identified by GC-MS. The methyl esters of DPA and epi-DPA were separated on TLC by multiple development in ethyl acetate-hexane (2:1). DPA was more polar than epi-DPA. DPA methyl ester (Me-DPA) and epi-DPA methyl ester (epi-Me-DPA) gave two separate peaks on GLC with retention times identical to those of the two products obtained by reduction of Me-PA with sodium borohydride[152]. The mass spectra of the four compounds were also very similar, showing molecular ions at m/e 296.

Epi-DPA was detected as one of the metabolites of endogenous ABA in dry bean seeds[151]. It was conclusively identified by GC-MS of its methyl ester.

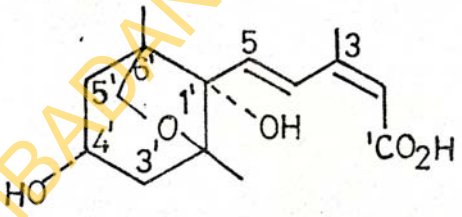
A probable metabolic pathway for ABA may be as follows:

ABA → 6'-hydroxymethyl ABA → PA → DPA and epi-DPA.



4'-dihydrophaseic acid

(CL XX I)



epi-4-dihydrophaseic acid

(CL XX II)

The richest sources of ABA and its metabolites in plants are fruit tissues.

Ethylene

The recognition of ethylene as a plant hormone can be traced back to the report in 1901 by Neljubow[153] that the abnormal growth (stunting, stem thickening and prostrate nature) of some seedlings in laboratory air was caused by traces of coal gas in the air. He identified ethylene and acetylene present in the coal gas as the active substances.

Gane[154] in 1934 demonstrated that ethylene was produced by plants. He passed the gaseous mixture from apple into tubes containing bromine at -65°C. The oil obtained was purified by fractional distillation. The presence of ethylene in the oil was demonstrated by the formation of N,N'-diphenyl ethylene diamine when the oil was heated with aniline. Since then it has been reported that ethylene is produced by many plants particularly during the ripening of fruits[155].

1. Physiological roles

Many physiological functions have been attributed to ethylene in plants[156,157]. Some of these are the following:

(a) breaking of dormancy,

(b) control of flower induction,

(c) induction of adventitious roots,

(d) promotion of the ripening process,

(e) closure of plumular hook,

(f) induction of epinasty,

(g) inhibition of leaf expansion,

(h) inhibition of extension growth, and

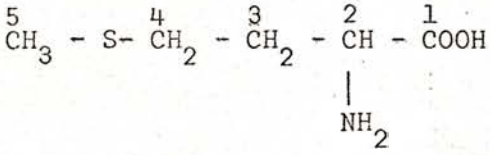
(i) acceleration of abscission.

Ethylene also has some effects on the physiology of IAA[156-158]. Generally ethylene reduces the level of IAA in plants. One of the means by which ethylene affects IAA level in plants is believed to be through the inhibition of the biosynthesis of IAA. This was particularly indicated by the work of Valdovinos et al[158]. They fed [^{14}C]-labelled tryptophan to cell-free preparations from stems of Coleus and Pisum. They found that enzymes from tissues treated with ethylene were much less active in producing labelled IAA and labelled CO_2 from the labelled tryptophan. Ethylene may also catalyse the destruction of IAA and inhibit IAA transport in some plants[156,157].

2. Biosynthesis

It is now believed that the amino-acid, methionine [CLXXIII] is the most likely precursor of ethylene in plants[159]. The possibility of methionine acting as a precursor of ethylene was first reported by Lieberman and Mapson[160]. They reported that ethylene was formed from copper catalysed breakdown of methionine. They also reported

that apple tissue slices fed with methionine produced more ethylene than controls



Methionine

[CLXXIII]

More significant evidence on the possibility of methionine acting as a precursor of ethylene was obtained through tracer studies. Lieberman et al[161a] added [^{14}C]-methionine labelled in carbon atoms 1, 2, 3, 4 or 5 to apple slices. They found that only methionine labelled in position 3 and 4 produced significant amounts of labelled ethylene. The work thus demonstrated that ethylene is derived from C-3 and C-4 of methionine.

The work in this thesis covers essentially the examination of some of the hormones in the extensively purified extracts obtained from two groups of cowpea fruits ('New Era' cultivar). These were 6-day old fruits from the lowest raceme and 2-day old fruits from the upper racemes.

RESULTS AND DISCUSSION

As stated in the introduction, the investigations of Ojehomon[1,2,4] seemed to implicate the presence of fruits which were older than five days, at the lowest raceme of the cowpea inflorescence, with the abscission of the fruitlets, and flowers of the upper racemes. The work of various other workers[8] had also revealed that the plant hormones are the principal endogenous factors that control abscission in plants.

It was therefore decided that a comparative analysis of some of the hormones in the maturing fruits of the lowest raceme and the abscising fruitlets of the upper racemes should be carried out. It was thought that a comparison of the hormonal contents of these two groups of fruits might lead to the understanding of some aspects of the endogenous factors controlling abscission in cowpea. This might then be of importance in the control of abscission in cowpea which is an important local source of protein.

The work in this thesis covers essentially the analysis of some of the hormones in immature cowpea fruits obtained from different racemes of the cowpea inflorescence.

The 'New Era' cultivar of cowpea was used. This cultivar is known to exhibit a high degree of abscission of flower buds, flowers and immature fruits[1-4].

Extracts were obtained from two groups of fruits. These were six-day old fruits from the lowest (first) raceme of the inflorescence and two-day old fruits from the upper racemes. The analysis of the extract obtained from fruits that were older than six days (from the lowest raceme) was also carried out for comparison.

The extraction procedure used[161b] for each group of fruits was carried out as described in the experimental section (page 215).

The analysis was concentrated on the acidic ethyl acetate (AE) fraction. This was because most of the free acidic plant hormones should be in the AE fraction.

Analysis of the acidic ethyl acetate fraction of the extract obtained from six-day old fruits

A portion of the acidic ethyl acetate (AE) fraction obtained from six-day old fruits was purified[162] with PVP.

In order to analyse the PVP purified acidic extract on gas-liquid chromatograph (GLC), suitable volatile derivatives had to be prepared. Aliquot of the purified extract was therefore methylated with ethereal diazomethane. The methyl ester thus obtained was silylated in pyridine with a mixture of hexamethyldisilazane and trimethylsilyl chloride.

A portion of the methyl ester trimethylsilyl ether (MeTMSi) derivative of the extract was then analysed on gas-liquid

chromatograph directly linked to a mass spectrometer (GC-MS).

The gas-liquid chromatogram of the MeTMSi derivative of the extract (Fig.6) had many peaks. No identification could easily be made directly from the mass spectra of most of the peaks. Most of the peaks appeared to be mixtures.

One of the peaks (Fig. 6, P38) however appeared to be a mixture of the methyl ester trimethylsilyl ether of gibberellin A_6 (MeA_6TMSi), [CLXXIV] and possibly $MeA_{17}TMSi$ [CLXXV] together with some impurities. The mass spectrum obtained from a scan of the first part of the peak had a molecular ion at m/e 432. There were prominent M^+-15(M^+-CH_3) and M^+-59(M^+-CH_3COO) ions. There were also prominent ions at m/e 303, 235, 208 and 207. The ions at m/e 303 and 235 were characteristic[39] prominent ions in the mass spectrum of MeA_6TMSi. The ions at m/e 208 and 207 were indicative[39] of hydroxylation at C-13. Although there were some other prominent ions (believed to be due to impurities) in the mass spectrum, the general fragmentation pattern was consistent with that of standard MeA_6TMSi[39].

The mass spectrum obtained from a scan of the middle of the peak (Fig. 6, P38), indicated that apart from MeA_6TMSi, $MeA_{17}TMSi$ might also be present. Apart from the ions mentioned above, there was an ion at m/e 492 which could be the molecular ion for

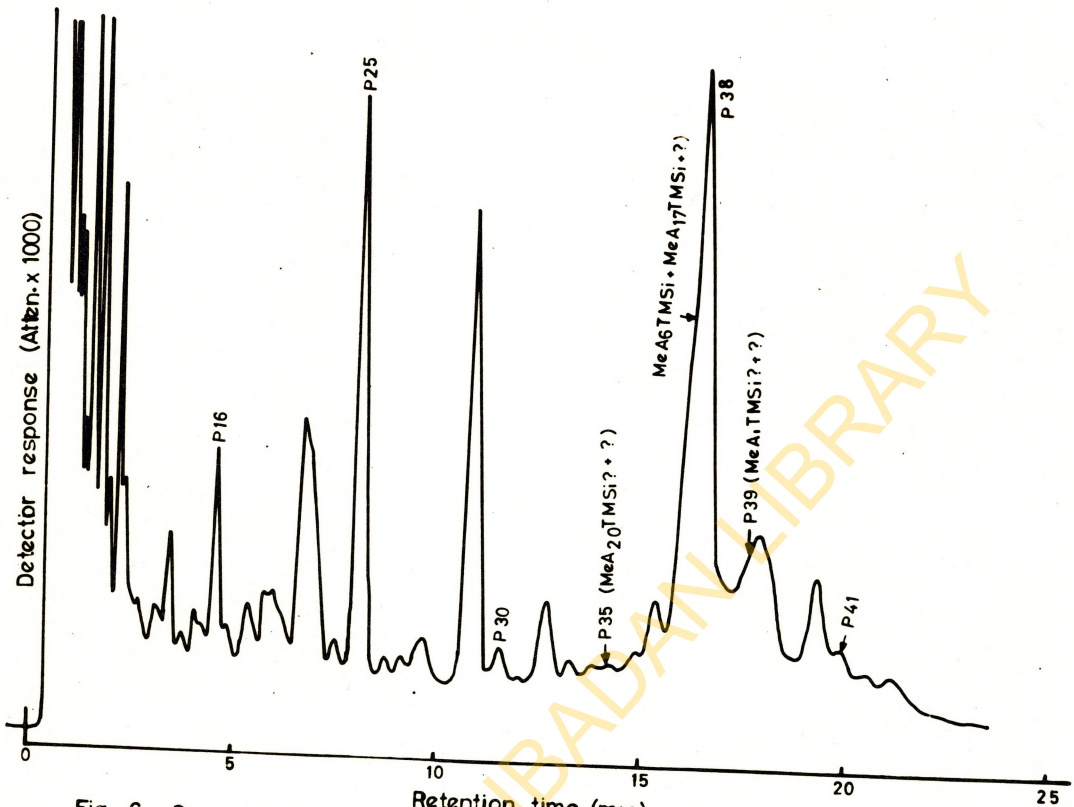


Fig. 6. Gas chromatogram of the MeTMSi derivative of AE extract from 6-day old fruits.
(2% SE 33, 130 x 0.4cm column, Nitrogen carrier gas 60ml/min, temperature 180 – 250° at 4°/min)

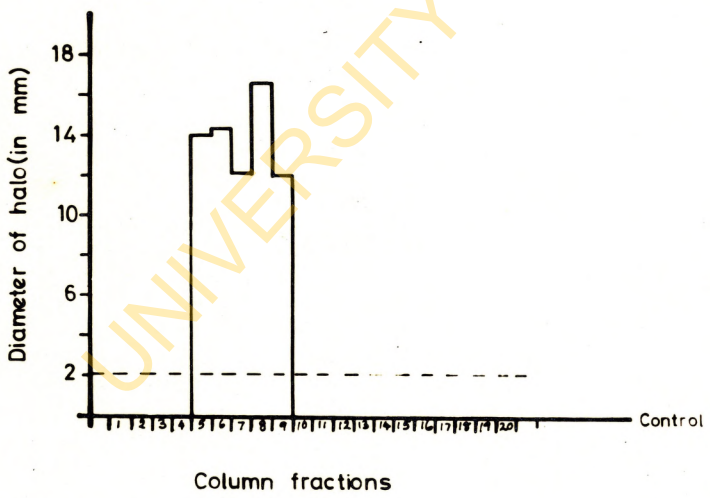
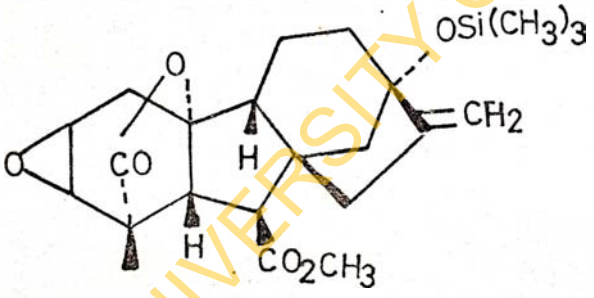


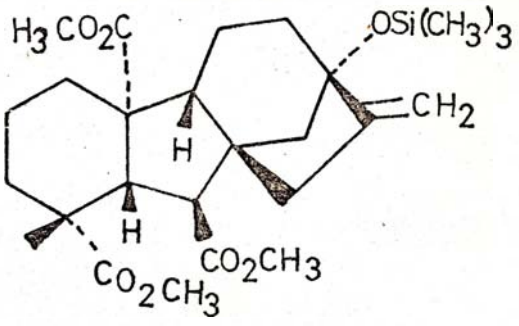
Fig. 7. Halo bioassay on column fractions of AE extract from 6-day old fruits.

$MeA_{17}TMSi$. There were also M^+-32 and M^+-119 ions. The M^+-119/120 ion had been reported[39] to be characteristic of C_{20}-GAs and was believed[39] to be due to the loss of two moles of acetic acid. This indicated the presence of at least two methoxycarbonyl groups in the molecule. The ions at m/e 207/208 might also be due to $MeA_{17}TMSi$ since GA_{17} has a hydroxyl group attached to C-13.

The peak (Fig. 6, P38) was therefore believed to consist of MeA_6TMSi and possibly $MeA_{17}TMSi$ together with some impurities.



MeA_6 TMSi
(CLXXIV)



MeA_{17} TMSi
(CLXXV)

The mass spectra of some of the other GLC peaks, (Fig. 6, P35, P39 and P41) indicated that some other GAs like GA_{20}, GA_1, and GA_8 might be present. Each of these mass spectra however had very many prominent ions which could not be accounted for. It was therefore difficult to make reasonable deductions from the mass spectra.

1. Column chromatography on AE fraction obtained from six-day old fruits.

On account of the large amount of impurities in the extract, it was decided to purify the extract further by charcoal-celite column chromatography. Twenty 150 cm^3 fractions were collected by gradient elution with increasing concentrations of acetone in water.

(a) TLC analysis of column fractions

Exploratory analytical thin-layer chromatography (TLC) was carried out on all the twenty column fractions.

The results of the analytical TLC indicated[136] that ABA and its metabolites might be present in fractions 4 and 5. The results also indicated[59] the probable presence of GAs in fractions 4 to 9.

(b) Barley half-seed (halo) bioassay of column fractions

The column fractions were tested for gibberellin activity by using the barley half-seed (halo) bioassay[163,164].

The results obtained from the halo bioassay are shown in Table 2 and the histogram is shown in figure 7. Gibberellin activity was observed in fractions 5 to 9.

TABLE 2

Results obtained in the halo bioassay carried out on the column fractions of the acidic extract obtained from six-day old fruits

Column Fractions	Diameter (in mm) of the halo for each half-seed										Total	Mean
	1	2	3	4	5	6	7	8	9	10		
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
5	16	15	14	12	16	14	12	13	14	14	140	14±1
6	16	15	14	16	17	14	13	13	12	13	143	14±2
7	15	9	14	15	7	13	11	10	13	14	121	12±3
8	16	17	16	18	16	17	18	15	18	15	166	17±1
9	15	15	12	11	13	12	11	9	11	11	120	12±2
10 to 20	0	0	0	0	0	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	0	0	0	0	0	0
Standard GA_3 2µg/cm^3	19	20	21	20	19	21	21	21	20	20	202	20±1

2. GC-MS analysis of selected column fractions

Both the analytical TLC and the bioassay results were used as a guide in selecting fractions that were to be examined on the GC-MS. The bioassay and TLC results indicated the probable presence of ABA and its metabolites in fractions 4 and 5, and gibberellins in fractions 4 to 9. It was therefore decided to examine fractions 4 to 10 on the GC-MS.

A portion of each column fraction was purified with PVP. Aliquots of the PVP purified fractions were methylated and then silylated. The derivatived extract of each column fraction was then examined on the GC-MS.

(a) Column fraction 4

The gas-liquid chromatogram of the methyl ester trimethylsilyl ether derivative of fraction 4 (figure 8) indicated the presence of many compounds. Some of the compounds that were identified in this fraction were the following:

(i) Methyl ester of phaseic acid (MePA) [CLXXVI]

The mass spectrum (Fig. 9) of the component (Fig. 8, P21) that was identified as MePA [CLXXVI] had a molecular ion at m/e 294 [CLXXVII]. There were prominent ions at m/e 276 (M^+-H_2O), 262 (M^+-MeOH) [CLXXVIII], 154 [CLXXIX], 153, 125 (side-chain) [CLXXX], 122 [CLXXXI], 121, 94 and 43 (CH_3CO^+). Some of these ions might arise[165] as shown in scheme 34 below.

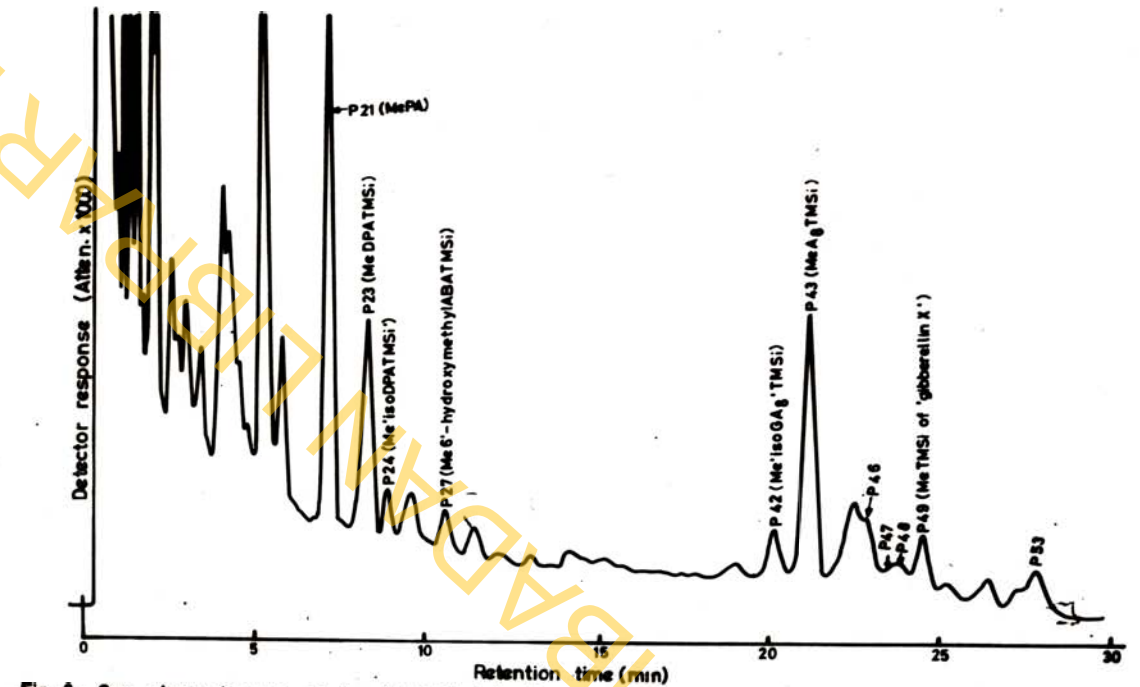


Fig. 8. Gas chromatogram of the MeTMSi derivative of column fraction 4 of the AE extract from 6-day old fruits.
(2% SE 33, 130 x 0.4 cm column, Nitrogen carrier gas 60 ml/min, temperature programme 180–250° at 4°/min)

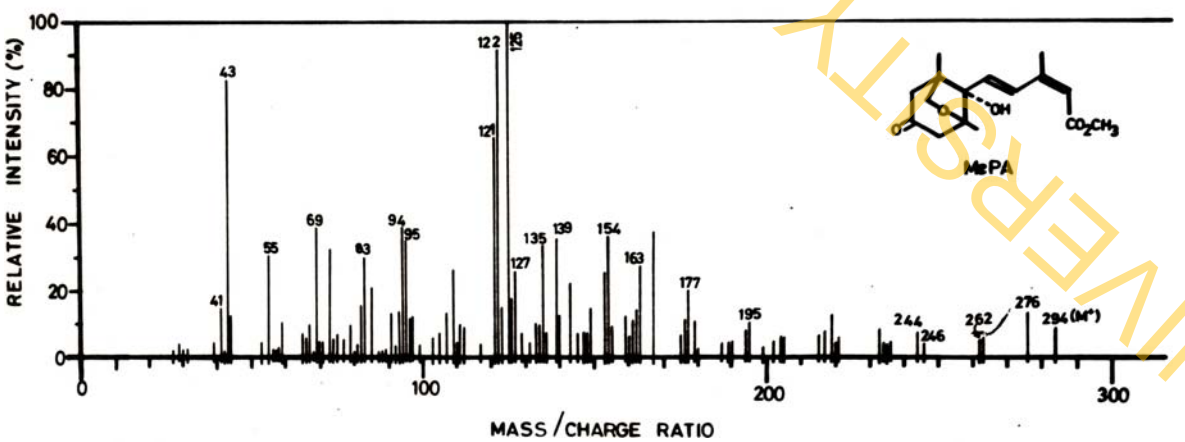
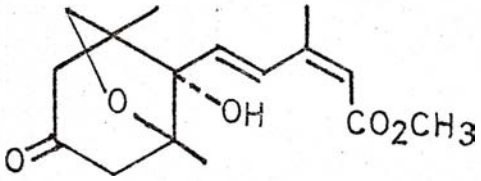
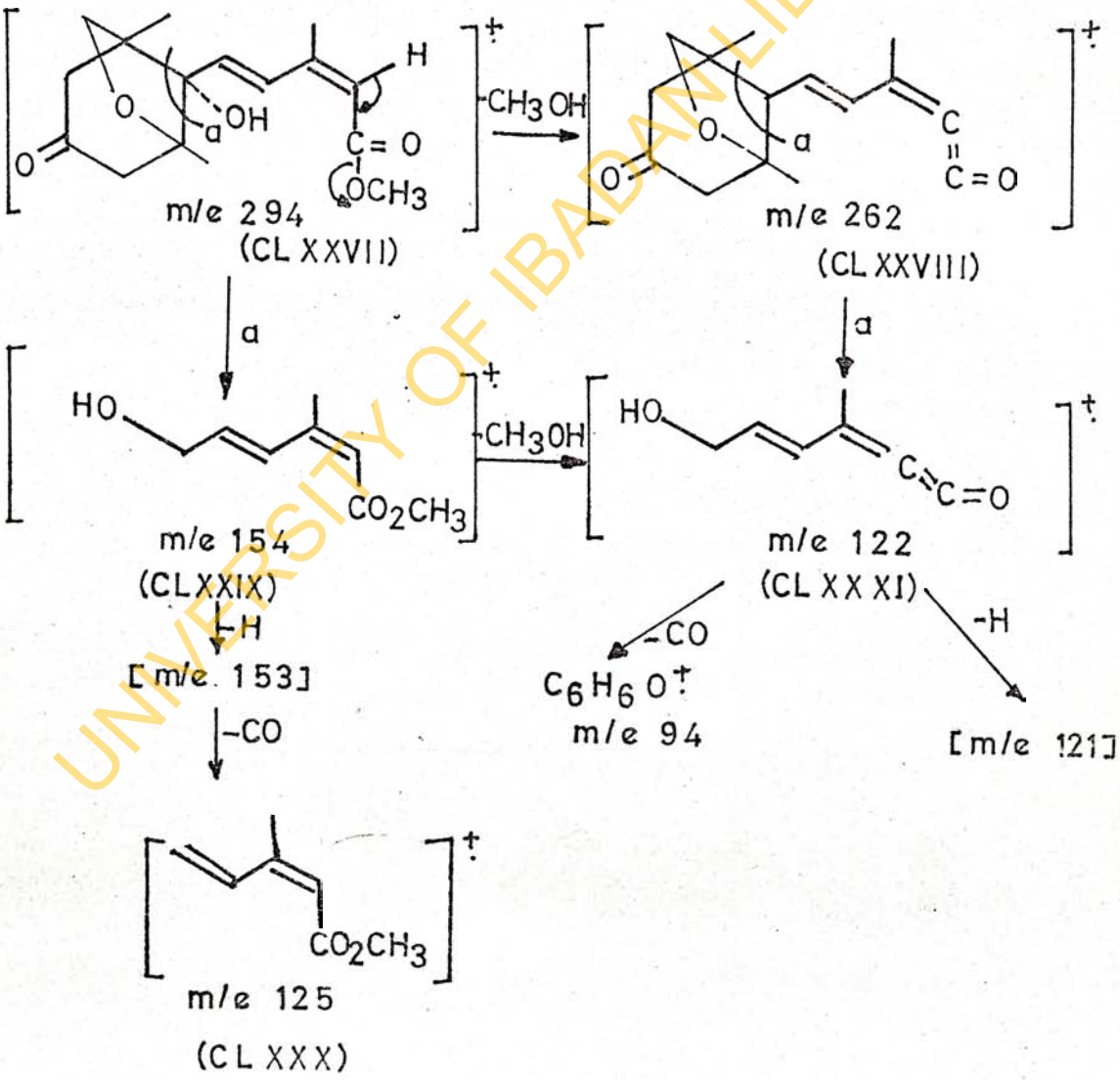


Fig. 9. Mass spectrum of MePA



Methyl ester of phaseic acid
(CLXXVI)

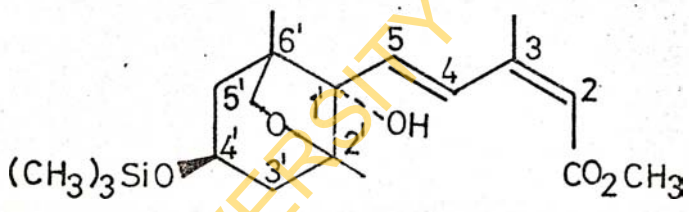
SCHEME 34



The mass spectrum (Fig. 9) was identical with that of standard MePA and with the published[165] mass spectrum of MePA. This thus indicated the presence of phaseic acid in this fraction.

(ii) Methyl ester trimethylsilyl ether of dihydrophaseic acid (MeDPATMSi) [CLXXXII]

The mass spectrum (Fig. 10) of the component (fig. 8, P23) that was identified as MeDPATMSi had a very weak molecular ion at m/e 368. There were the ions at m/e 73 $[(CH_3)_3Si]$ and m/e 75 associated[39] with the trimethylsilyl (TMSi) group.



MeDPA TMSi
(CLXXXII)

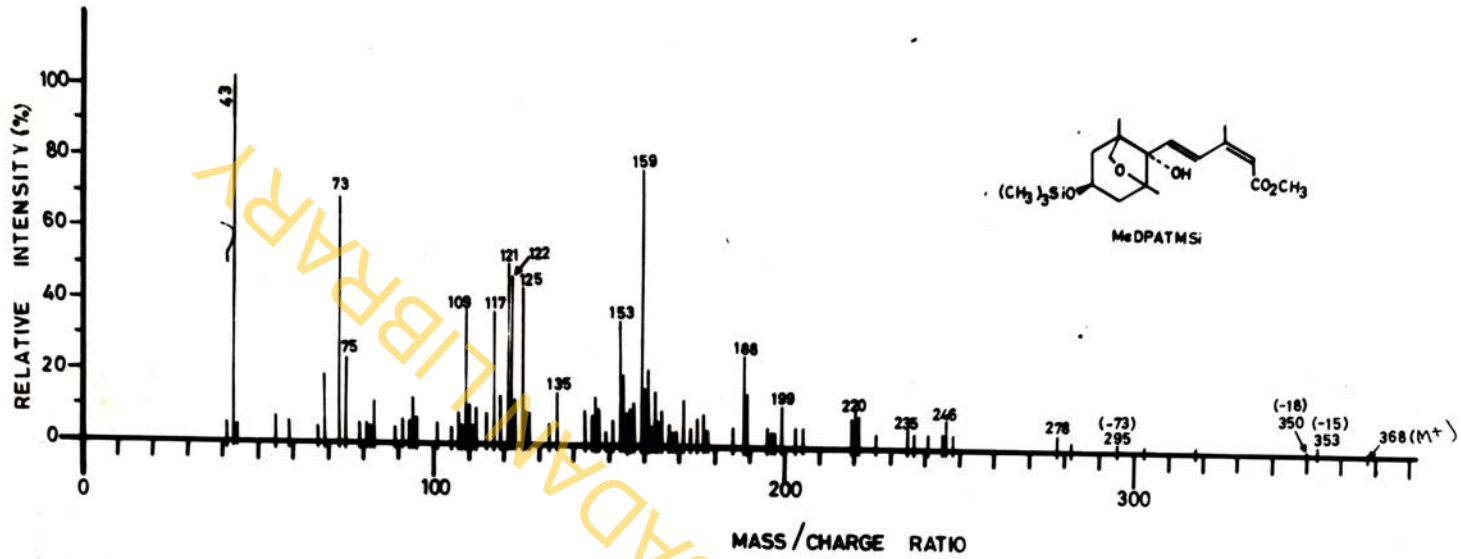


Fig. 10. Mass spectrum of MeTMSi ether of DPA

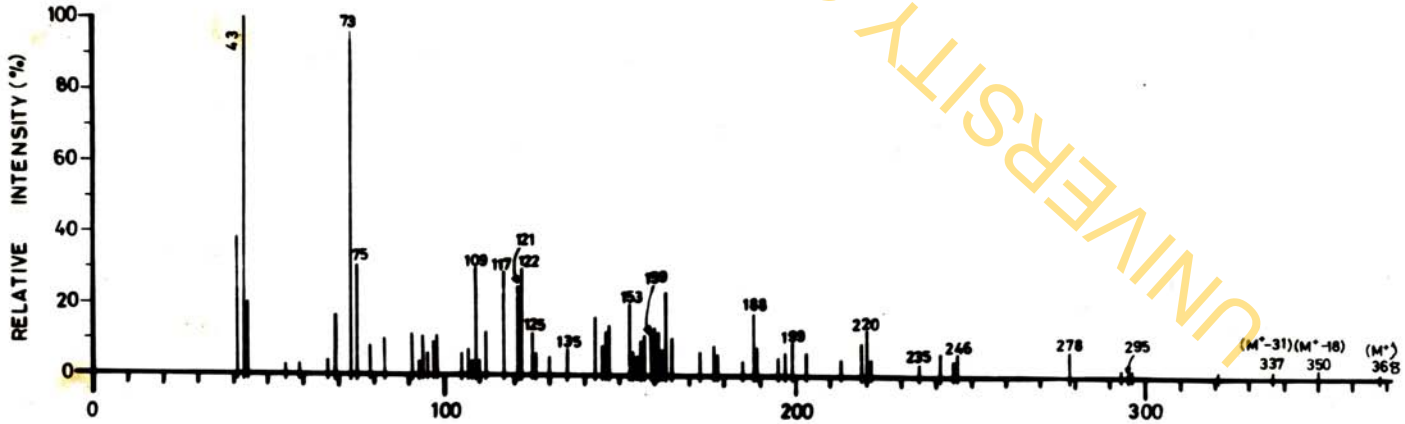


Fig. 11. Mass spectrum of the MeTMSi ether of 'isoDPA'

There were significant ions at m/e 295 [M^+-$(CH_3)_3Si$], 278 [M^+-$(CH_3)_3SiOH$], 246 [M^+-$(CH_3)_3SiOH$,CH_3OH], and m/e 220 [M^+-$(CH_3)_3SiO$, CH_3COO]. There were other prominent ions at m/e 188, 159, 154, 153, 125, 122, 121, 117 and 109.

The ions at m/e 154, 153, 125, 122 and 121 could arise from the molecular ion just like in the case of MePA shown in shceme 34 above.

Just as in the spectrum of MePA, there was a very prominent ion at m/e 43 (base peak) which was believed to be CH_3CO^+.

The mass spectrum (Fig. 10) was identical with that of authentic MeDPATMSi. It was also very similar to the mass spectrum that was reported for MeDPATMSi by Zeevart and Milborrow[151].

(iii) Methyl ester trimethylsilyl ether of isomer of dihydrophaseic acid (iso-MeDPATMSi).

The mass spectrum (Fig. 11) obtained from a scan of the component (Fig. 8, P24) that was identified as iso-MeDPATMSi had a molecular ion at m/e 368.

The compound had a longer retention time than MeDPATMSi.

The general fragmentation pattern of the compound was similar to that of MeDPATMSi. The relative intensities of some of the ions however differed significantly. For example the ion at m/e 159 which was the second most prominent ion (76%) in the mass spectrum of MeDPATMSi had a relative intensity of only 11 per cent in the

mass spectrum of this compound. Also the ions at m/e 121, 122, 125 and 135 had much lower intensities than in the mass spectrum of MeDPATMSi.

The mass spectral data of the 4'-epimer of MeDPATMSi had been reported[151] in literature. The relative intensities reported for some of the ions in the mass spectrum of the 4'-epimer of MeDPATMSi differed significantly from the relative intensities of these ions in this compound. The compound was therefore not considered to be the 4'-epimer of MeDPATMSi.

The mass spectrum of the 2-trans isomer of MeDPATMSi has not been published and no standard sample was available. It was therefore not possible to compare the mass spectrum of the compound with that of the 2-trans isomer of MeDPATMSi.

Conclusive identification of the compound was therefore not possible from the mass spectrum alone. It was however decided to call it an isomer of MeDPATMSi since the mass spectrum was very similar to that of MeDPATMSi.

(iv) Methyl ester trimethylsilyl ether of 6'-hydroxymethylABA [CLXXXIII]

The mass spectrum (Fig. 12) obtained from a scan of the component (Fig. 8, P27) which was believed to be the methyl ester trimethylsilyl ether of 6'-hydroxymethyl abscisic acid [CLXXXIII] had a molecular ion at m/e 366.

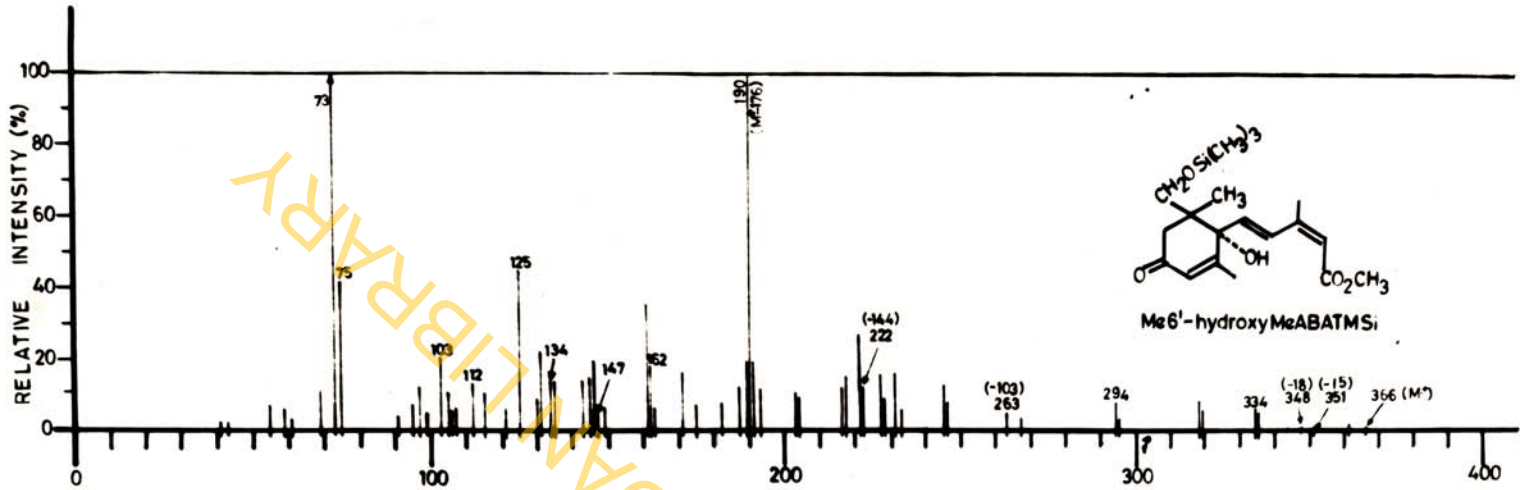


Fig.12 Mass spectrum of MeTMSi ether of 6'-hydroxymethylABA

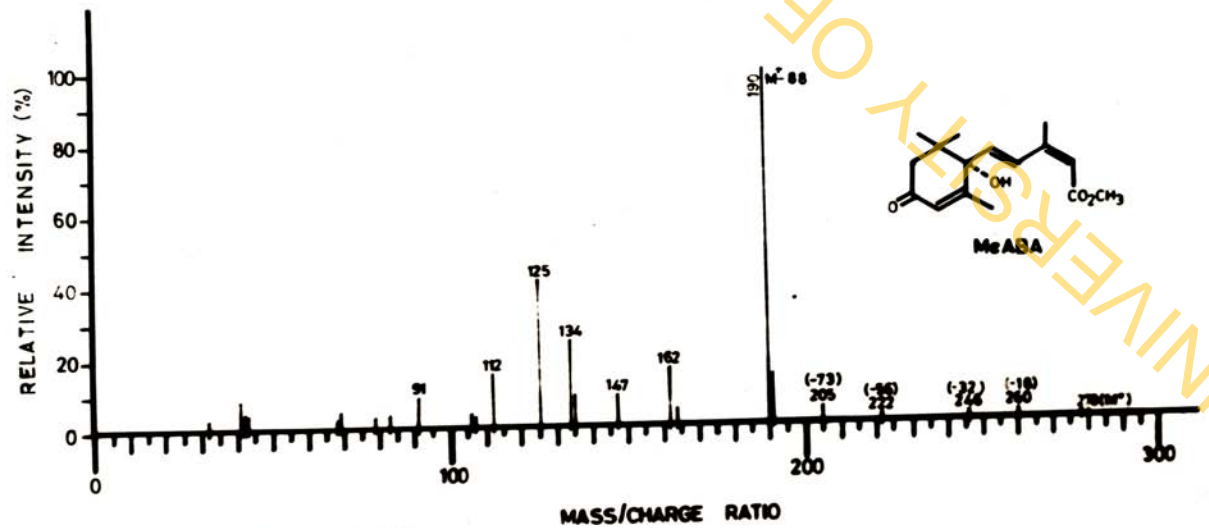
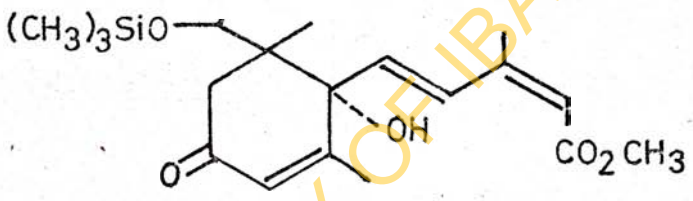


Fig.13 Mass spectrum of MeABA

The first part of the mass spectrum of the compound had some resemblance to the mass spectrum (Fig. 13) of the methyl ester of ABA. Just as in the mass spectrum of MeABA, there was a base peak at m/e 190. Also the ions at m/e 162, 147, 134, 125, 112 and 91 which were prominent in the mass spectrum of MeABA were also prominent in the mass spectrum of the compound. These seemed to indicate some structural similarities between the compound and MeABA.



Me 6'-hydroxymethyl ABA TMSi

(CLXXXIII)

The presence of the ions at m/e 73 and m/e 75 which were associated with the TMSi group indicated that the compound was silylated. The tertiary hydroxyl group of ABA was normally resistant to silylation. It could not normally have been silylated by the method employed which involved adding a silylating mixture

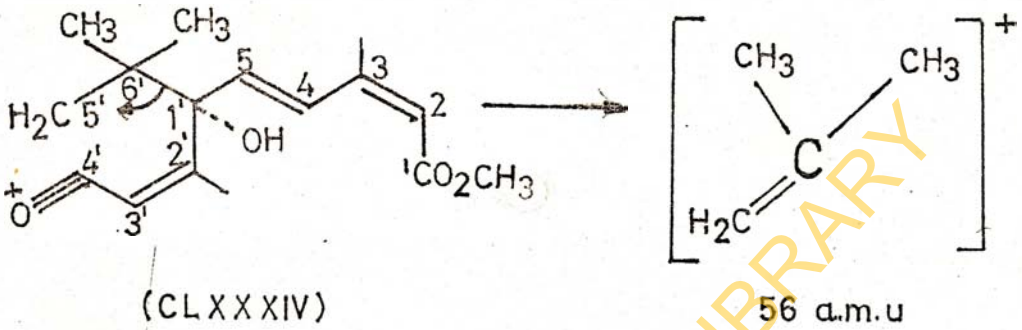
of hexamethyldisilazane, trimethylsilyl chloride, and pyridine (2:1:2) to the methylated extract in pyridine. The fact that the compound was easily silylated thus indicated that there was another hydroxyl group somewhere in the molecule which was the one that was silylated. This indication was confirmed by the fact that the molecular ion of the compound was at m/e 366. This suggested that the compound could be derived from MeABA (M^+ 278) by the replacement of a hydrogen atom by the trimethylsilyl ether [$(CH_3)_3Si$-O] group (89 a.m.u.) This indicated that before derivatization the compound would be hydroxyabscisic acid.

There was a prominent ion at m/e 103 which was believed to be $-CH_2OSi(CH_3)_3$ indicating the presence of methylene hydroxyl (CH_2OH) group in the underivatized molecule. There was also an M^+-103 ion at m/e 263.

The problem left was to deduce the position of hydroxylation. The position of hydroxylation was deduced to be one of the two methyl groups attached to C-6' as described below.

A significant M^+-56 ion occurred[130] in the mass spectrum of MeABA or ABA. This was reported[130] to be due to the loss of isobutylene [CLXXXV] by a cleavage of the ring in the MeABA molecular ion [CLXXIV] as shown[130] in scheme 35 below.

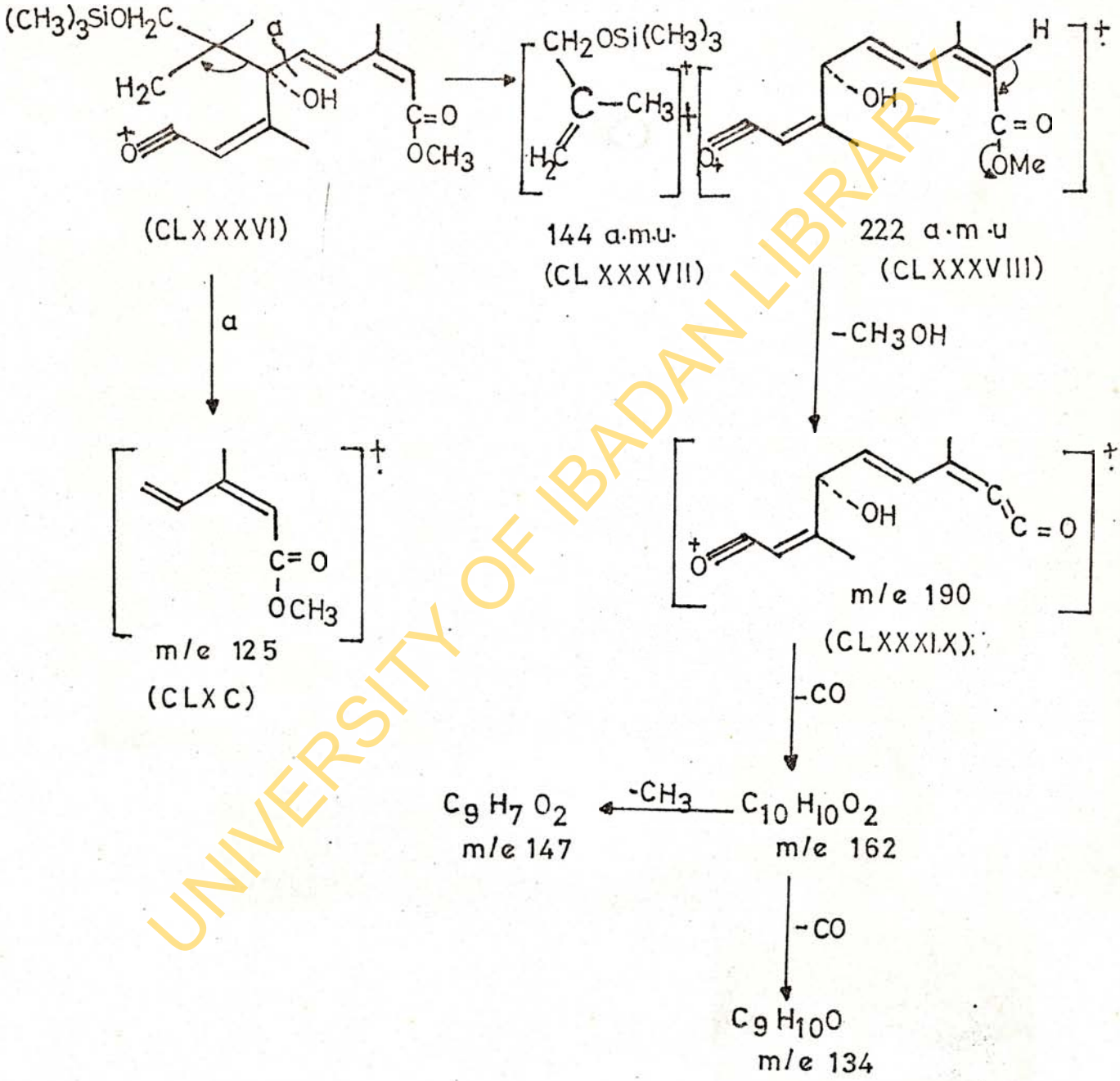
SCHEME 35



(CLXXXIV) 56 a.m.u (CLXXXV)

If it was one of the gem dimethyl groups attached to C-6' that was hydroxylated, the M^+-56 ion should be replaced by an M^+-144 ion. This followed from the fact that the loss of isobutylene [CLXXXV] (56 a.m.u.) would be replaced by the loss of the silylated group [CLXXXVII], (144 a.m.u.) as shown in scheme 36 below. This replacement of the M^+-56 ion by an M^+-144 ion was observed in the mass spectrum of the compound. It had an M^+-144 ion [CLXXXVIII] at m/e 222 and there was no M^+-56 ion. The M^+-144 ion was believed to be derived from the molecular ion [CLXXXVI] through the loss of [CLXXXVII].

SCHEME 36



In the mass spectrum of MeABA, the base peak at m/e 190 was reported[130] to be due to the loss of isobutylene and methanol from the parent ion. Similarly in the mass spectrum of this compound, the loss of the ion [CLXXVII] and methanol (from the methoxycarbonyl group) gave the base peak at m/e 190 [CLXXXIX].

Just as in the case of MeABA, the successive losses of CO from the base peak would give rise to the ions at m/e 162 and m/e 134. The loss of a methyl group from the ion at m/e 162 would give rise to the ion at m/e 147. The cleavage of the side-chain as shown above in scheme 36 would give rise to the m/e 125 ion [CLXC].

The fragmentation pattern of the mass spectrum of this compound thus agreed very well with the expected fragmentation pattern of the methyl ester trimethylsilyl ether of 6'-hydroxymethyl abscisic acid [CLXXXIII]. Unfortunately there was no standard sample of 6'-hydroxymethyl ABA which could have been used as a reference. The evidence from the mass spectrum was however considered to be strong enough for the identification.

6'-hydroxymethyl ABA was first identified by Milborrow[147] as a metabolite of labelled ABA fed to tomato (Lycopersicon exculentum) shoots. It was originally referred to as 'metabolite C' of ABA and was later identified[147] as 6'-hydroxymethyl ABA as described in the introduction (page ١١٦).

'Metabolite C' was reported[147] to be very unstable, rearranging easily to phaseic acid. It was reported[139] that the mass spectrum of 'metabolite C' could not be obtained because the compound rearranged to phaseic acid in the mass spectrometer. Subsequent reported[139] attempts to isolate 6'-hydroxymethyl ABA were unsuccessful.

It was possible in this case to obtain the mass spectrum of the MeTMSi derivative of the 6'-hydroxymethyl ABA probably because the TMSi group protected the 6'-hydroxymethyl group. This would thus prevent the nucleophilic attack of the 6'-hydroxymethyl on C-2' which could have led to the formation of phaseic acid methyl ester.

This is the first identification of naturally occurring 6'-hydroxymethyl abscisic acid.

(v) Methyl ester trimethylsilyl ether of isomer of gibberellin A_8

The mass spectrum (Fig. 14) obtained from a scan of peak P42, Fig.8, showed the general fragmentation pattern[39] for the MeTMSi derivative of C_{19}-gibberellins.

The mass spectrum had a general resemblance to the mass spectrum (Fig. 15) of the MeTMSi derivative of gibberellin A_8 [CLXCI]. The retention time was however shorter than that of MeA_8TMSi.

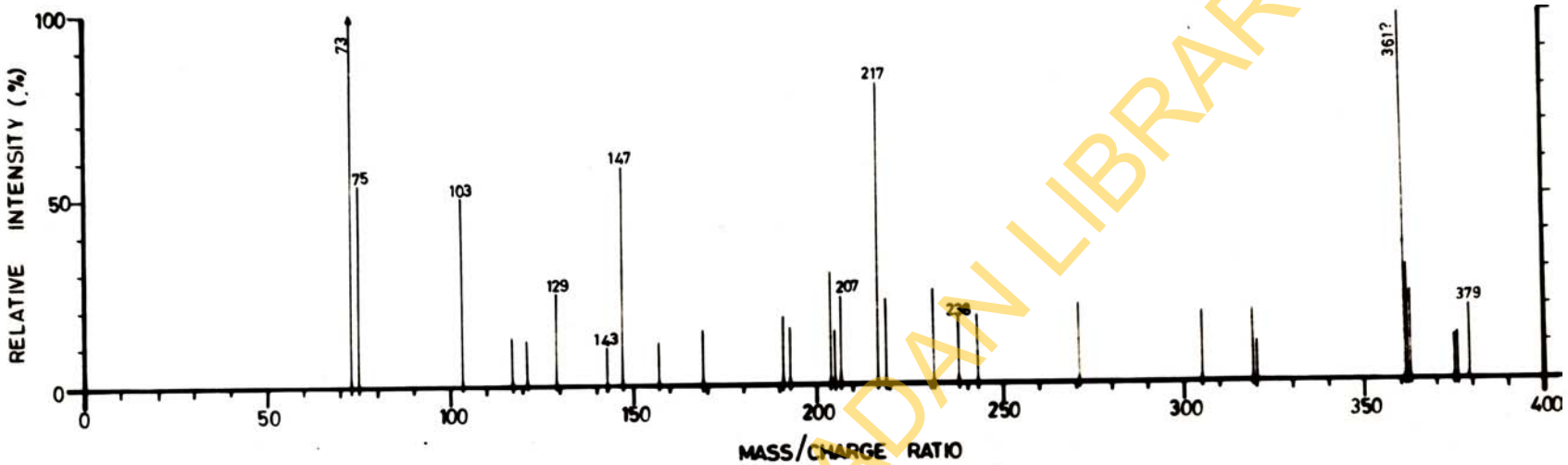


Fig. 14 Mass spectrum of the MeTMSi ether of 'isoGA8'

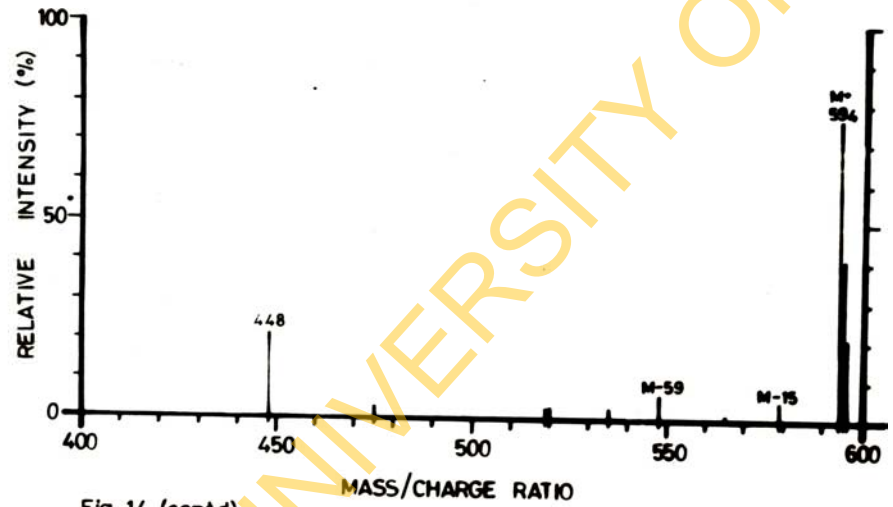


Fig. 14 (contd)

There were M^+-15 (M^+-CH_3), M^+-89 [M^+-$OSi(CH_3)_3$] ions as well as the ions at m/e 73 and 75 associated with the trimethylsilyl ether group. There was also the M^+-59 (M^+-CH_3COO) ion associated with the loss of the methoxycarbonyl group.

The ion at m/e 129, characteristic of 3-hydroxylation was present. Also present were the ions at m/e 147 and m/e 217 associated with the presence of the 2,3-diol system.

The compound might therefore differ from GA_8 only in the position of the third hydroxyl group. In GA_8, the third hydroxyl group is attached to C-13. In the mass spectra of the MeTMSi derivatives of gibberellins, C-13 hydroxylation was usually indicated[39] by prominent ions at m/e 207/208 and by the fact that either one of these ions or the molecular ion was normally the base peak. Although the ions at m/e 207/208 were present in the mass spectrum of this compound, their relative intensities were low when compared with the relative intensities of the ions at m/e 147 and 217. Moreover neither the 207/208 ions nor the molecular ion was the base peak. The third hydroxyl group might therefore be attached to a different carbon atom instead of C-13 as in GA_8.

The prominent ion at m/e 361 could not be accounted for and might be due to impurities.

Conclusive identification of the compound was not possible from the mass spectrum alone. It was therefore just decided to refer to it tentatively as an isomer of gibberellin A_8.

(vi) Methyl ester trimethylsilyl ether of gibberellin A_8

The mass spectrum (Fig. 15) that was obtained from a scan of peak P43, Fig. 8, had a molecular ion at m/e 594 (base peak).

The ions at M^+-15 (M^+-CH_3), M^+-90 [M^+-$(CH_3)_3SiOH$], m/e 73 and m/e 75 associated with the trimethylsilyl ether group were present.

Other prominent ions that were present included the ion at m/e 129 due to 3-hydroxylation and the ions at m/e 147 and 217 indicative of the 2,3-diol system. The ions at m/e 207 and 208 associated with the presence of 13-hydroxyl were also present.

The compound was conclusively identified as the methyl ester trimethylsilyl ether of gibberellin A_8 (MeA_8TMSi) [CLXCI] because its mass spectrum was identical with the mass spectrum of standard MeA_8TMSi and with the published[39] mass spectrum of MeA_8TMSi.

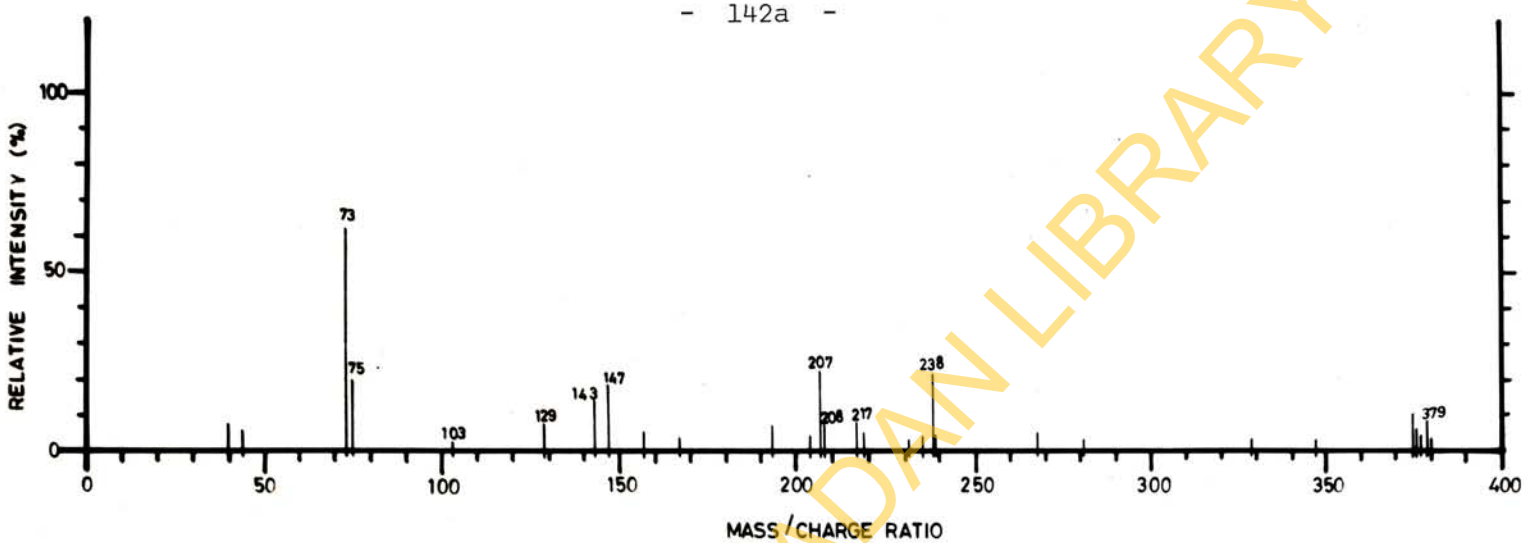


Fig. 15 Mass spectrum of MeA$_8$TMSi

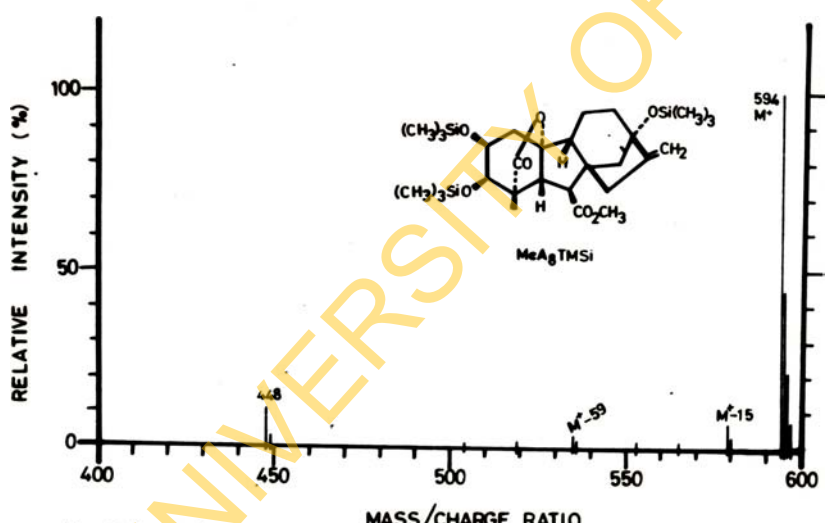
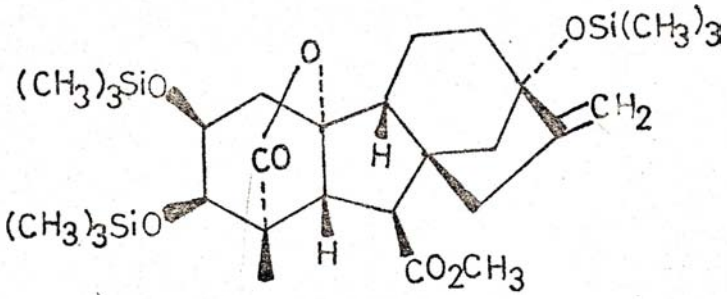


Fig. 15 (contd)



MeA8TMSi

(CLXCl)

(vii) Methyl ester trimethylsilyl ether of gibberellin X

The mass spectrum (Fig. 16) of the component (P49, Fig. 8) had a molecular ion at m/e 684, and a very prominent M^+-103 ion (base peak). The presence of this prominent M^+-103 ion and an m/e 103 ion was indicative[39] of the presence of a $-CH_2OSi(CH_3)_3$ group in the molecule.

There were prominent ions at m/e 129, 147 and 217 indicating the presence of hydroxyl groups at the 2,3-positions.

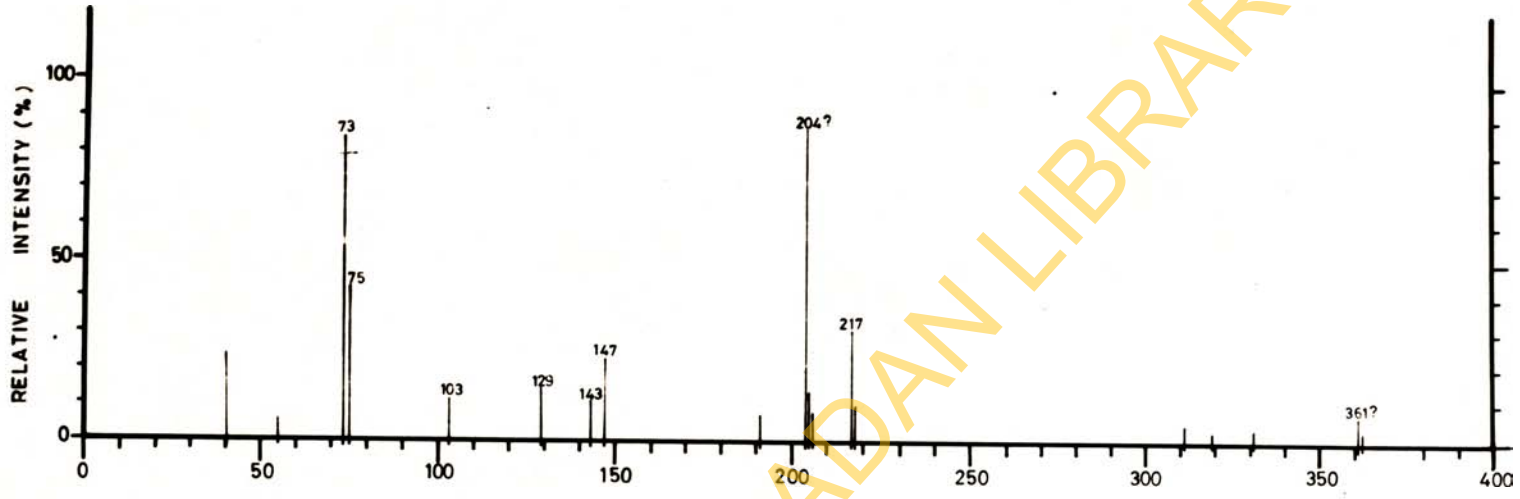


Fig 16 Mass spectrum of MeTMSi ether of 'gibberellin X'

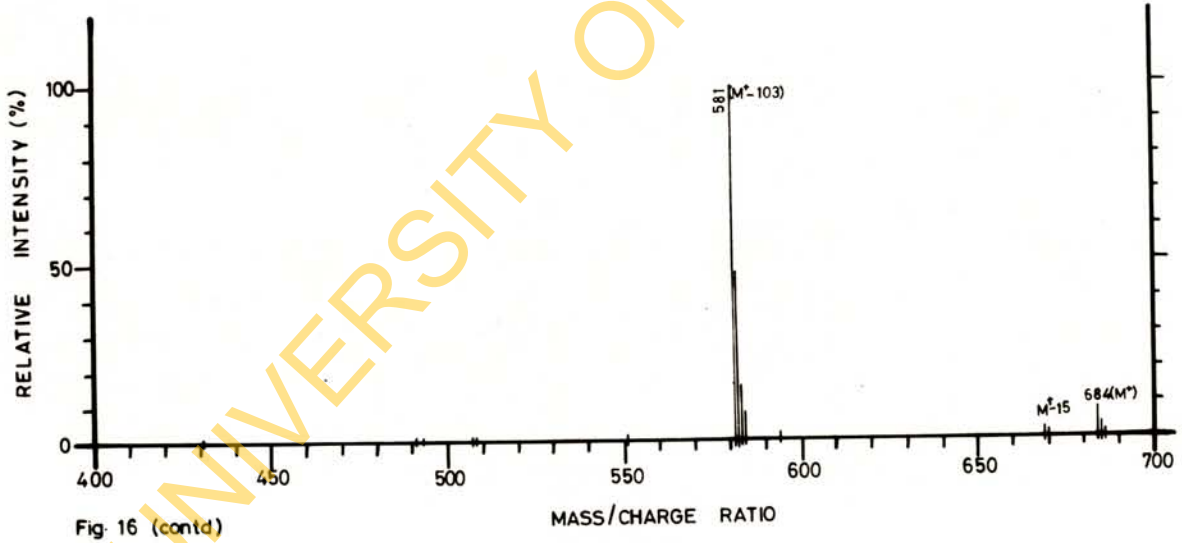


Fig 16 (contd.)

The absence of prominent ions at m/e 207/208 coupled with the fact that the molecular ion at m/e 684 was not very prominent indicated the absence of a hydroxyl group in the 13-position.

The absence of M^+-119/120 ions (due to the loss of 2 moles of CH_3COOH) and M^+-91/92 ions (due to the loss of 59/60 a.m.u. from one methoxycarbonyl group and 31/32 a.m.u. from another methoxycarbonyl group) indicated that the compound was monocarboxylic.

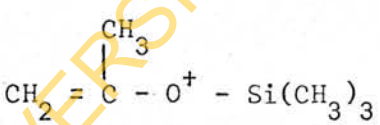
The mass spectrum of the compound showed the general pattern associated with the mass spectra[39] of MeTMSi derivatives of C_{19}-gibberellins. The mass spectrum was however different from the mass spectrum of the MeTMSi derivative of any of the known C_{19}-gibberellins. It was therefore believed to be a new gibberellin and was tentatively referred to as gibberellin X.

The molecular weight of the MeTMSi derivative of this gibberellin indicated the presence of four hydroxyl groups in the molecule. Two of these should be in the 2,3-positions as discussed above. One other hydroxyl group should be a CH_2OH group as discussed above. It could be either the C-18 methyl group or the C-17 methylene group that had been converted to the hydroxymethyl group.

The likely sites left for the fourth hydroxyl group were positions 11, 12, 15 and 16. 15-hydroxylation was usually characterized[166] by a prominent ion at m/e 156. 11- and 12-

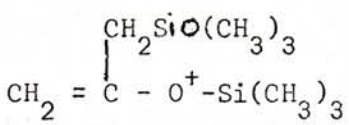
hydroxylated GAs generally showed[166] prominent M^+-90 ions (due to the loss of TMSiOH from the parent ion). The absence of prominent ions at m/e 156 and M^+-90 in the mass spectrum of the compound therefore suggested the absence of hydroxyl group at position 11, 12 or 15.

The C-16 was therefore left as the most probable site for the fourth hydroxyl group. Normally 16-hydroxylation was indicated[39] by the presence of a prominent ion [XXIX] at m/e 130 which was usually the base peak. This ion was derived from cleavage of ring D (Scheme 8 page 40). This ion was absent in the mass spectrum of this compound. This was thought to be due to the fact that the C-17 was also hydroxylated. Thus the methyl group in the m/e 130 ion would be replaced by the $CH_2OSi(CH_3)_3$ group as shown in [CLXCII]. Therefore no m/e 130 ion should be observed



m/e 130

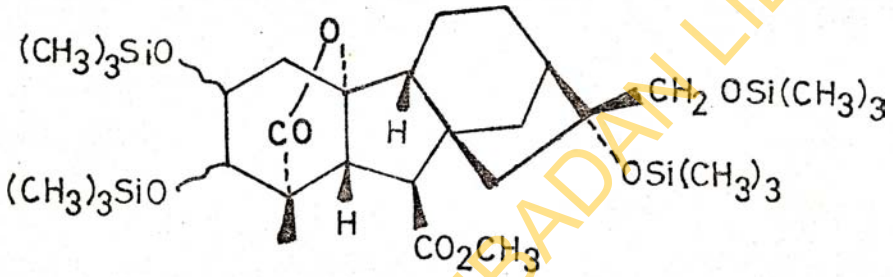
[XXIX]



[CLXCII]

The most likely structure of the compound was therefore considered to be the methyl ester trimethylsilyl ether of 2, 3, 16, 17-tetrahydroxy gibberellin A_9 [CLXCIII]. The stereochemistry of the 2,3-hydroxyl groups was not certain. Although the stereo-

chemistry of the 16-hydroxyl group was also not certain, the α configuration was thought to be the more likely by comparison with the structures of the known gibberellins.



(CLXCIII)

An attempt was made to confirm the structure postulated by partial synthesis from gibberellin A_4. The results obtained will be discussed later in this chapter.

(b) Column fraction 5

The gas-liquid chromatogram of the MeTMSi derivative of this fraction (Fig. 17) had many peaks. This indicated that there

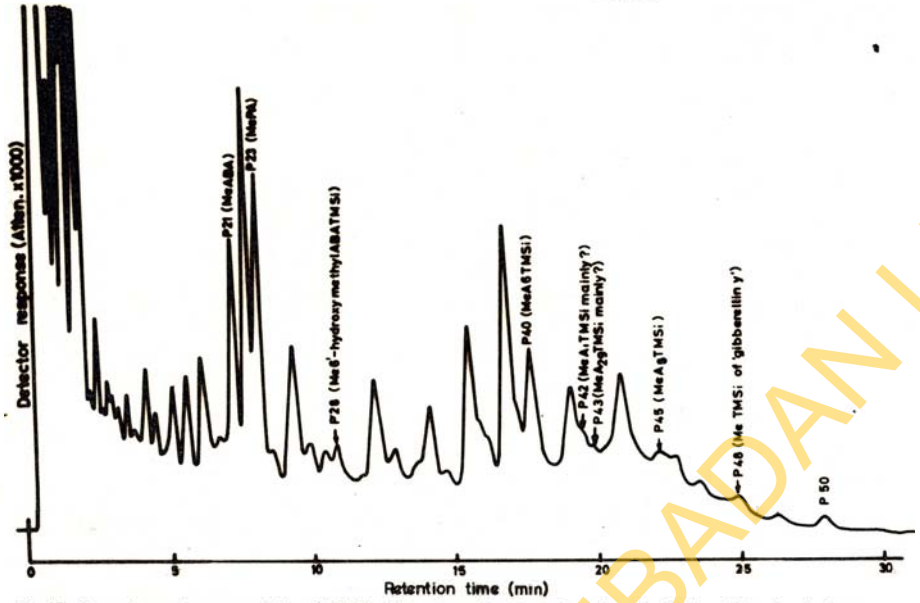


Fig-17. Gas chromatogram of the MeTMSi derivative of column fraction 5 of the AE extract from 6-day old fruits.
(2% SE. 130 x 0·4 cm column. Nitrogen carrier gas 60 ml/min. temperature programme 180-250° at 4°/min.)

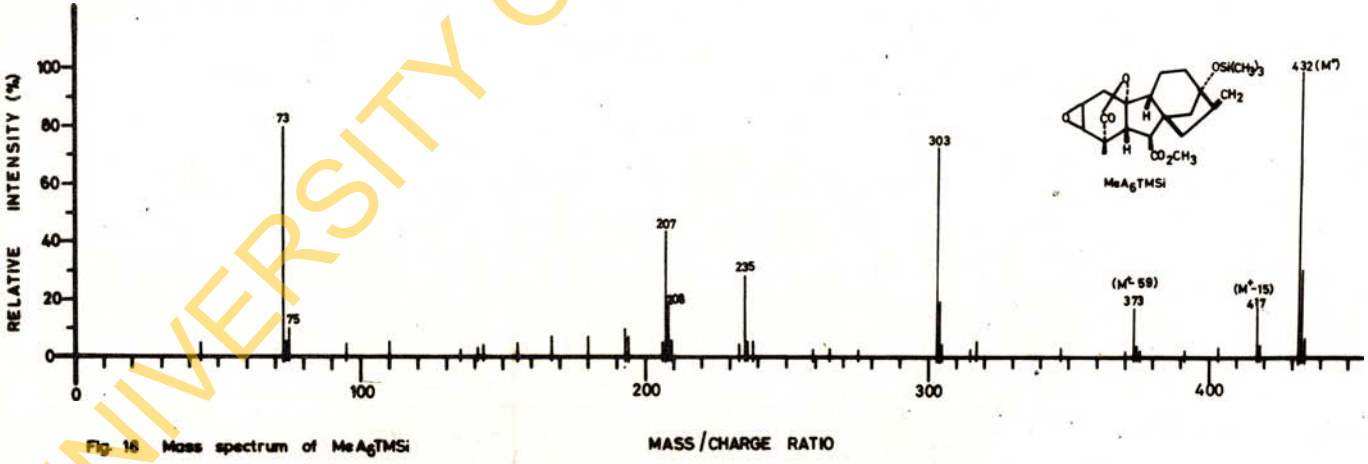


Fig. 18 Mass spectrum of MeA6TMSi

were many compounds present. Most of these compounds could not be identified and were thought to be mostly phenolic impurities.

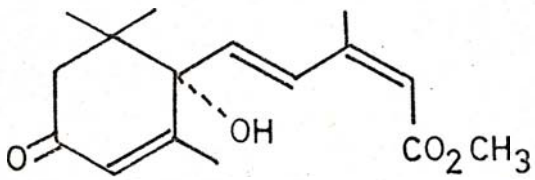
Some compounds which were identified earlier in fraction 4 were also present in this fraction. These were (i) methyl ester of phaseic acid [CLXXVI] (P23, Fig. 17), (ii) methyl ester trimethylsilyl ether of 6'hydroxymethyl abscisic acid [CLXXXIII] (P28, Fig. 17) and (iii) methyl ester trimethylsilyl ether of gibberellin A_8 [CLXCI] (P45, Fig. 17).

Some other compounds which were identified in this fraction are listed below.

(i) Methyl ester of abscisic acid [CLXCIV]

The mass spectrum (Fig. 13) of the component (P21, Fig. 17) had a weak molecular ion at m/e 278. There were important ions at M^+-18, M^+-32, M^+-56, m/e 190 (base peak), m/e 162, m/e 147, m/e 134, m/e 125 and m/e 112.

The M^+-18 and the M^+-32 ions were due to the loss of H_2O and CH_3OH respectively from the molecular ion. The other ions (M^+-56, m/e 190, m/e 147, m/e 134 and m/e 125) were derived as explained earlier (Scheme 36, page 138).



MeABA

(CLXCIV)

The mass spectrum was identical to that of authentic MeABA and was identical to the published[167] mass spectrum of MeABA. It was therefore conclusively identified as MeABA.

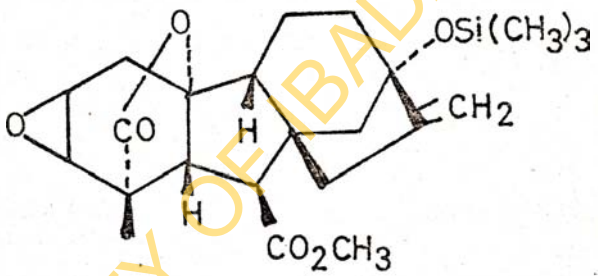
(ii) Methyl ester trimethylsilyl ether of gibberellin A_6 (MeA_6TMSi) [CLXXIV]

The mass spectrum (Fig. 18) of the component (P40, Fig. 17) had a strong molecular ion (base peak) at m/e 432. This coupled with the presence of prominent ions at m/e 207/208 indicated the presence of a hydroxyl group in the 13-position.

There were the ions at m/e 73, m/e 75 and M^+-15 associated with the TMSi ether group.

There were other prominent ions at M^+-59 (M^+-CH_3COO), m/e 303 and m/e 235.

The mass spectrum was identical with that of standard MeA_6TMSi and with the published[39] mass spectrum of MeA_6TMSi. The compound was therefore conclusively identified as methyl ester trimethylsilyl ether of gibberellin A_6 [CLXXIV].



CLXXIV

MeA_6TMSi

(iii) Methyl ester trimethylsilyl ether of gibberellin Y

The mass spectrum (Fig. 19) of the component (P48, Fig. 17) had a very strong molecular ion (base peak) at m/e 596.

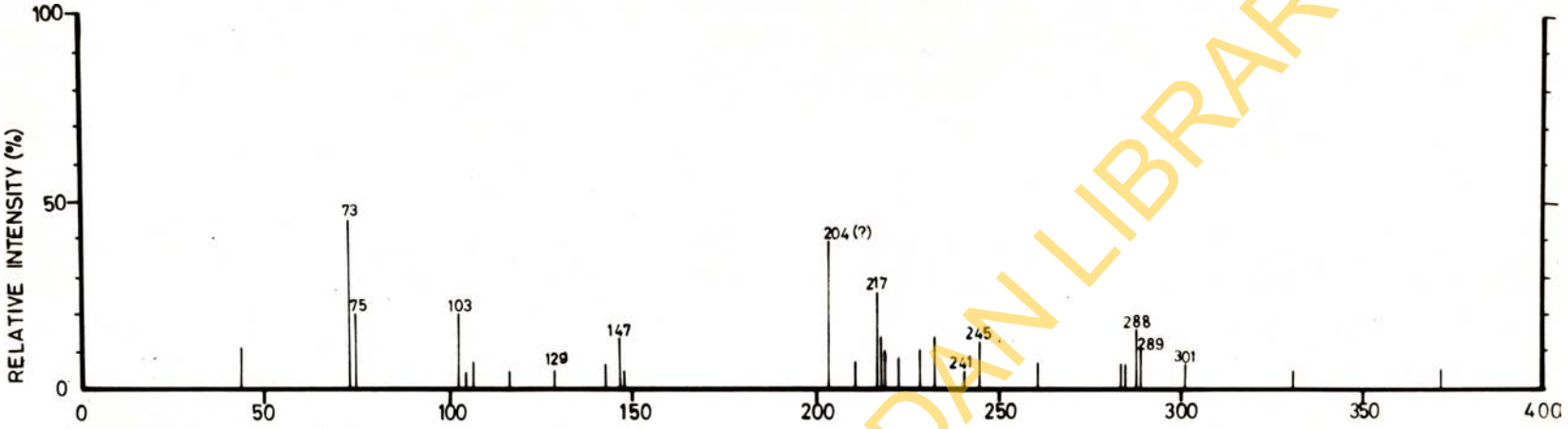


Fig. 19. Mass spectrum of MeTMSi derivate of 'gibberellin Y'

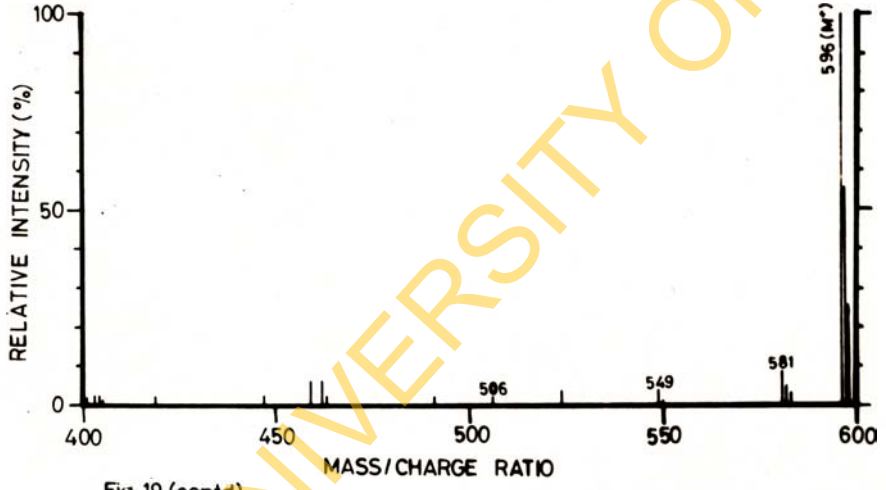


Fig. 19 (contd)

There were prominent ions at m/e 129 (indicating 3-hydroxylation), m/e 147 and m/e 217 (indicating 2,3-diol system).

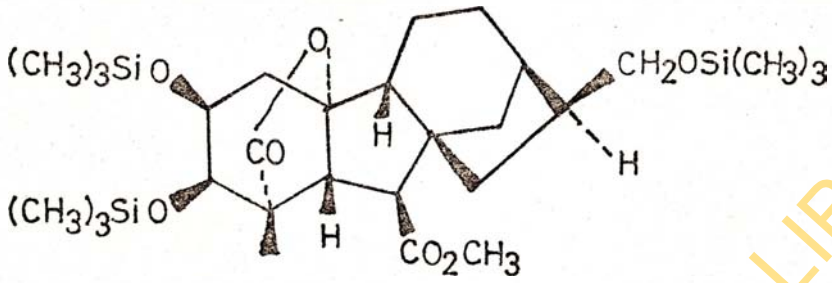
There was a prominent ion at m/e 103. This was taken to indicate the presence of $CH_2OSi(CH_3)_3$ (103 a.m.u.) group in the molecule.

There were no m/e 207/208 ions. 13-hydroxylation was therefore considered to be absent.

The mass spectrum of the compound had the general characteristic features of the mass spectra[39] of MeTMSi derivatives of C_{19}-gibberellins. It was however different from the mass spectra of the MeTMSi derivatives of the known gibberellins. It was therefore believed to be a new gibberellin and was tentatively referred to as gibberellin Y.

The most likely structure of the compound was considered to be methyl ester trimethylsilyl ether of 2,3,17-trihydroxy gibberellin A_9 [CLXCV].

The stereochemistry of the 2- and 3- hydroxyl groups were not certain. 2β, 3β-hydroxylation was however thought to be the most likely by analogy with the known gibberellins.



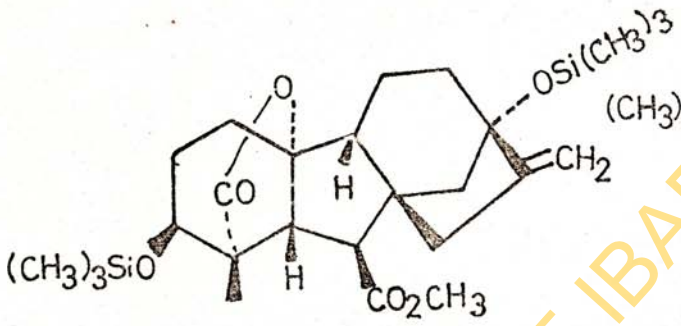
(CLXCV)

An attempt was made to confirm this postulated structure by partial synthesis from gibberellin A_4. The results obtained will be discussed later in this chapter.

Methyl ester trimethylsilyl ether of gibberellin A_1 (MeA_1TMSi) [CLXCVI] and methyl ester trimethylsilyl ether of gibberellin A_{29} ($MeA_{29}TMSi$) [CLXCVII] were also thought to be probably present in fraction 5.

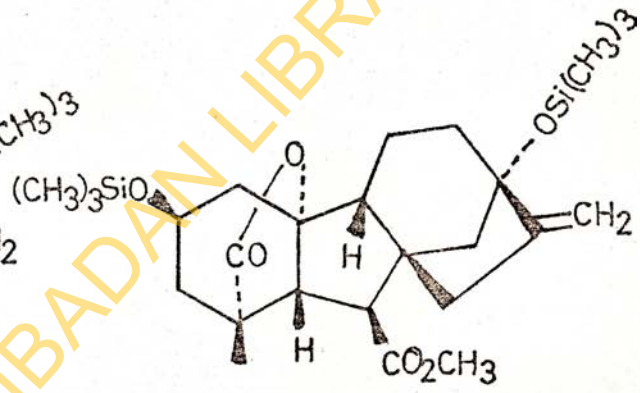
The mass spectrum of the peak (P42, Fig. 17) that was thought to contain MeA_1TMSi had a prominent molecular ion at m/e 506. All the significant ions in the mass spectrum[39] of

MeA_1TMSi were present. There were however many other ions present which could not be due to MeA_1TMSi. The presence of MeA_1TMSi was therefore not certain at this stage, although it was thought to be likely.



$MeA_1TMSi(M^+ 506)$

(CLXCVI)



$MeA_{29}TMSi(M^+ 506)$

(CLXCVII)

The mass spectrum of the peak (P43, Fig. 17) which was thought to be probably mainly $MeA_{29}TMSi$ also had a molecular ion at m/e 506. All the significant ions in the mass spectrum[168] of $MeA_{29}TMSi$ were present. Definite identification as $MeA_{29}TMSi$ was however impossible because of the presence in the mass spectrum

of many prominent ions which could not be due to $MeA_{29}TMSi$.

(c) Column fraction 6

None of the components in the gas-liquid chromatogram of the MeTMSi derivative of fraction 6 could be identified from the mass spectra obtained. Most of them were thought to be phenolic impurities.

(d) Column fraction 7

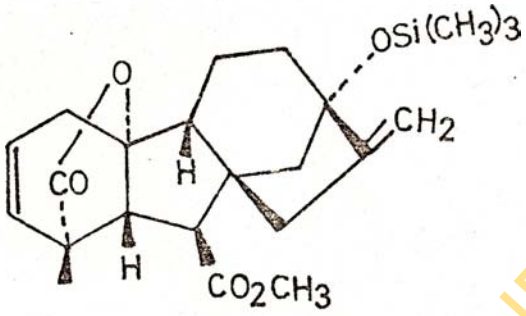
All the peaks in the gas-liquid chromatogram of this fraction except one could not be identified from their mass spectra.

One of the peaks however appeared to contain a mixture of MeTMSi derivatives of two gibberellins together with some impurities.

In the mass spectrum of the peak, there were some prominent ions at m/e 416 (base peak), m/e 417, m/e 418, and m/e 419. Their relative intensities were 100, 42, 58 and 25 respectively. The pattern of the relative intensities of these ions seemed to indicate that the mass spectrum might be that of a mixture. The ion at m/e 416 might then be the molecular ion of one of the components of the mixture while the ion at m/e 418 might be the molecular ion of another component.

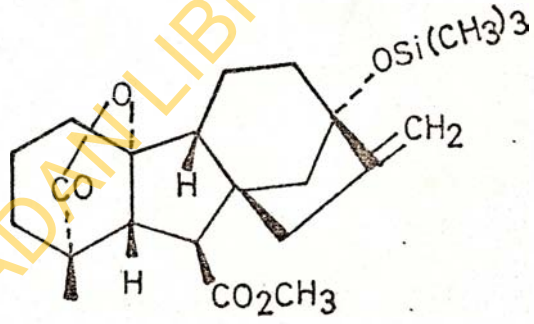
The component with M^+ 416 was considered to be probably MeA_5TMSi [CLXCVIII]. This was because of the presence of other ions

at m/e 401 (M^+-CH_3), 372 (M^+-CO_2), 343, 299, 275, 208, 207 and 180 which are important ions in the mass spectrum[39] of MeA_5TMSi.



MeA_5TMSi

(CLXCVIII)



$MeA_{20}TMSi$

(CLXCIX)

The other component with molecular ion at m/e 418 was considered to be probably $MeA_{20}TMSi$ [CLXCIX]. This was because some other important ions in the mass spectrum[39] of $MeA_{20}TMSi$ were also present. These were the ions at m/e 403 (M^+-CH_3), m/e 375, m/e 359 (M^+-CH_3COO),

m/e 314, m/e 301, m/e 208 and m/e 207.

Conclusive identification of these two gibberellins was however not possible at this stage on account of the presence of a large number of other prominent ions in the mass spectrum which could not have been derived from either MeA_5TMSi or $MeA_{20}TMSi$.

(e) Column fraction 3

The mass spectra obtained from a scan of most of the peaks in the gas-liquid chromatogram of the MeTMSi derivative of this fraction could not be interpreted.

One of the peaks however was believed to contain the MeTMSi derivative of a gibberellin together with some impurities.

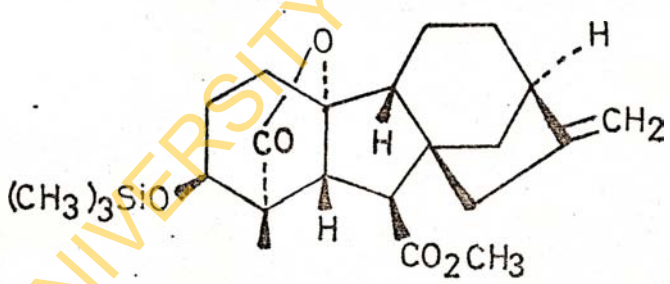
The mass spectrum that was obtained from a scan of the peak had a molecular ion at m/e 418. There were M^+-15, M^+-18, M^+-32, M^+-60 and M^+-90 ions. There were also very prominent M^+-129, M^+-134, M^+-193 and M^+-194 ions. All these ions were important prominent ions in the mass spectrum[39] of standard MeA_4TMSi [CC].

The M^+-134, M^+-193 and M^+-194 ions were particularly characteristic of the mass spectra[39] of MeA_4TMSi and MeA_7TMSi. The M^+-134 ion had been shown[39] to be $C_{15}H_{28}O_3Si$ and the M^+-193/194 ions to be $C_{13}H_{24/25}OSi$ in the case of MeA_4TMSi. These fragment

ions should contain ring A of MeA_4TMSi.

Also present in the mass spectrum of the peak were the ions at m/e 129 (indicative of 3-hydroxylation) and m/e 73/75 (from the TMSi group).

Although there were many other ions which could not have been due to MeA_4TMSi in the mass spectrum, the evidence outlined above was thought to be strong enough to indicate the presence of MeA_4TMSi [CC] in the peak.



MeA_4TMSi
(CC)

(f) Column fractions 9 and 10

None of the many peaks in the gas-liquid chromatograms of the MeTMSi derivatives of these two fractions could be identified from their mass spectra.

The results obtained above from the GC-MS analysis of the selected derivatized column fractions (of the acidic ethyl acetate extract obtained from six-day old fruits) showed that a lot of impurities still interfered seriously with the analysis. Only fraction 4 out of the fractions analysed was reasonably free from impurities. For example, although the 'halo' bioassay results (Fig. 7 and Table 2) indicated the presence of gibberellin activity in fractions 6 and 9, no gibberellin was detected in these two fractions by GC-MS analysis. This was presumably due to the fact that the impurities present in these fractions were masking the presence of the gibberellins. Further purification of the column fractions was therefore considered to be necessary.

(3) TLC of selected column fractions and GC-MS of some Rf zones of the TLC

An attempt was made to find out whether further purification of some of the column fractions by TLC would help to remove some of the impurities in the fractions.

Aliquots of the PVP purified extract obtained from column fractions 5 to 10 were mixed together and subjected to TLC. The thin-layer chromatogram was divided into ten equal zones after development in ethyl acetate/chloroform/acetic acid (15: 5: 1, v/v/v).

'Halo' bioassay indicated gibberellin activity in the extract obtained from Rf 0.4 - 0.5 and 0.5 - 0.6, of the thin-layer chromatogram.

Extracts from selected Rf zones (0.3 - 0.4, 0.4 - 0.5, and 0.5 - 0.6) were analysed on the GC-MS as the MeTMSi derivatives. The result of the 'halo' bioassay was taken into consideration in selecting these Rf zones. Also most of the gibberellins and some of the other plant hormones such as ABA and PA would be expected to occur within the selected Rf zones in the developing mixture that was used. The results obtained in the GC-MS analysis of the derivatized selected Rf zones are given below.

(a) Rf 0.3 to 0.4

No gibberellin could be identified in the MeTMSi derivative

of the extract obtained from Rf 0.3 to 0.4. The fragmentation pattern of the mass spectra of the various peaks in the gas-liquid chromatogram indicated that a lot of impurities were still present.

(b) Rf 0.4 to 0.5

No particular gibberellin could be conclusively identified in the MeTMSi derivative of the extract from Rf 0.4 to 0.5. Some of the mass spectra obtained showed the fragmentation pattern associated with the MeTMSi derivatives of some gibberellins, for example MeA_1TMSi. Each of these mass spectra however also contained many ions which could not be due to the methyl esters or the MeTMSi of gibberellins. Conclusive identification of any gibberellin in this Rf zone was therefore not possible.

(c) Rf 0.5 - 0.6

The GC-MS analysis of the MeTMSi derivative of the extract from Rf 0.5 to 0.6 afforded the identification of some plant hormones in this Rf zone.

One of the peaks in the gas-liquid chromatogram (Fig. 20) of the MeTMSi derivative of the extract from this Rf zone appeared to consist of a mixture of the MeTMSi derivatives of two gibberellins and some impurities. The mass spectrum that was obtained from a scan of the first part of the peak (P19, Fig. 20)

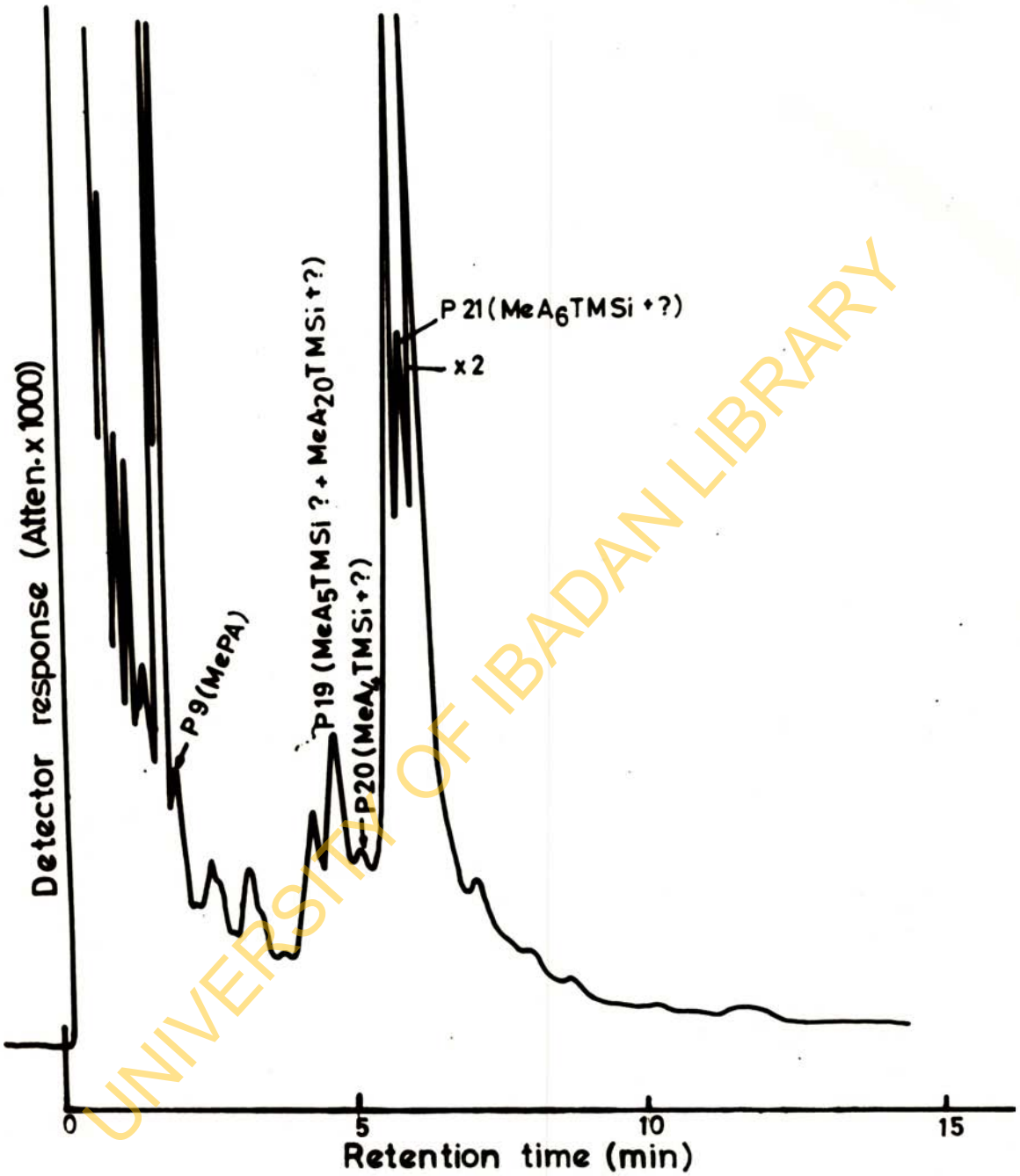


Fig. 20. Gas chromatogram of TLC Rf. 0·5-0·6 of fractions 5 to 10 of AE extract from 6-day old fruits (MeTMSi derivative)
(2% SE 33, 130x0·4cm column, Nitrogen carrier gas 60ml/min, temperature programme 180-250° at 4°/min)

showed a molecular ion at m/e 418. There were some other prominent ions at m/e 403 (M^+-CH_3), 375, 359 (M^+-CH_3COO), 301, 235 and m/e 208/207. This fragmentation pattern was similar[39] to that of $MeA_{20}TMSi$. It was thought that the evidence was strong enough to indicate the presence of $MeA_{20}TMSi$ despite the presence of other prominent ions in the mass spectrum.

The mass spectrum obtained from a scan of the centre of the peak indicated the presence of $MeA_{20}TMSi$ and possibly MeA_5TMSi together with some impurities.

The mass spectrum of the last part of the peak appeared to consist largely of impurities and possibly MeA_5TMSi. There was a molecular ion at m/e 416. Other ions that were indicative of the likely presence of MeA_5TMSi were at m/e 401 (M^+-CH_3), 372, 357 (M^+-CH_3COO), 299, 208 and 207. The presence of many other prominent ions which could not be due to MeA_5TMSi in the mass spectrum, made conclusive identification of MeA_5TMSi impossible. It was however thought that the presence of MeA_5TMSi was very likely.

Also identified in the MeTMSi derivative of the extract from Rf 0.5 - 0.6 were MeA_4TMSi [CC], MeA_6TMSi [CLXXIV] and MePA [CLXXVI].

On the whole it appeared that the further purification of the column fractions on TLC did not significantly remove the impurities that were interfering with the GC-MS analysis.

Several plant hormones were thus identified (by means of GC-MS analysis) in the acidic ethyl acetate extract from 6-day old fruits. These were GA_4, GA_6, GA_8, 'iso'GA_8, GA_{20} and two new gibberellins tentatively called gibberellins X and Y. Others were ABA, PA, DPA, 'iso'DPA and 6'-hydroxymethylABA. Also probabl present were GA_1, GA_5, GA_{17} and GA_{29}. They were identified in some of the fractions that were collected during the charcoal-celite column chromatography of the acidic ethyl acetate extract that was obtained from 6-day old fruits as shown in table 3 below.

TABLE 3

Plant hormones identified in 6-day old fruits of Vigna unguiculata (L.Walp) by GC-MS

Column fractions	Eluant [%$(CH_3)_2CO$ in H_2O]	Compounds
4	25 - 38	PA, DPA, 'iso'DPA, 6'-hydroxymethylABA, GA_8, 'iso'GA_8 and gibberellin X
5	38 - 44	PA, 6'-hydroxymethylABA, ABA, GA_6, GA_8, gibberellin Y, GA_1? and GA_{29}?
7	49 - 53	GA_5? and GA_{20}
8	53 - 58	GA_4

(4) Wheat coleoptile segment bioassay on the acidic ethyl acetate extract that was obtained from 6-day old fruits

(a) Bioassay on TLC Rf zones

A portion of the PVP purified acidic ethyl acetate extract from 6-day old fruits was subjected to thin-layer chromatography using EtOAc/$CHCl_3$/HOAc (15: 5: 1) as the developing solvent mixture. The thin-layer chromatogram was divided into ten equal zones. Aliquots of the extract from each Rf zone were tested for biological activity in the wheat coleoptile segment bioassay.

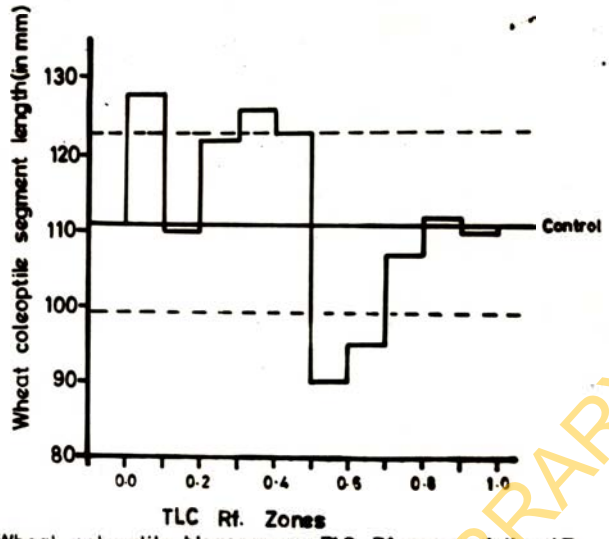
The wheat coleoptile segment bioassay is basically a test for auxins and growth inhibitors. Gibberellins do not generally respond to the test. This is because gibberellins do not generally have any reasonable promotive effect on the growth extension of plant sections although they are very effective in promoting growth extension of intact plants. The main aim of the test was to find out if there was any active auxin in the extract.

The results that were obtained in the bioassay are shown in Table 4 below and the histogram is shown in Figure 21.

TABLE 4

Results obtained in the wheat coleoptile segment bioassay on TLC Rf Zones of the acidic ethyl acetate extract from 6-day old fruits

TLC Rf Zones	Length of wheat coleoptile segments (in mm)											
	1	2	3	4	5	6	7	8	9	10	TOTAL	MEAN
0.0-0.1	131	125	129	121	134	128	120	130	132	130	1280	128±5
0.1-0.2	120	102	102	121	109	105	109	115	115	106	1104	110±7
0.2-0.3	140	132	116	110	115	130	120	118	115	126	1222	122±9
0.3-0.4	130	130	131	120	116	120	140	120	124	126	1257	126±7
0.4-0.5	118	130	130	122	132	113	113	126	127	116	1227	123±8
0.5-0.6	94	89	93	83	90	90	86	92	93	89	899	90±3
0.6-0.7	90	100	94	98	94	95	92	98	94	96	951	95±3
0.7-0.8	113	108	103	109	104	110	105	102	108	110	1072	107±4
0.8-0.9	113	104	110	115	120	114	108	110	118	110	1122	112±5
0.9-1.0	110	110	110	115	105	108	110	114	106	112	1100	110±3
CONTROL	104	110	119	106	116	110	105	115	115	110	1110	111±5



Fig·21· Wheat coleoptile bioassay on TLC Rf zones of the AE fraction from 6-day old fruits.

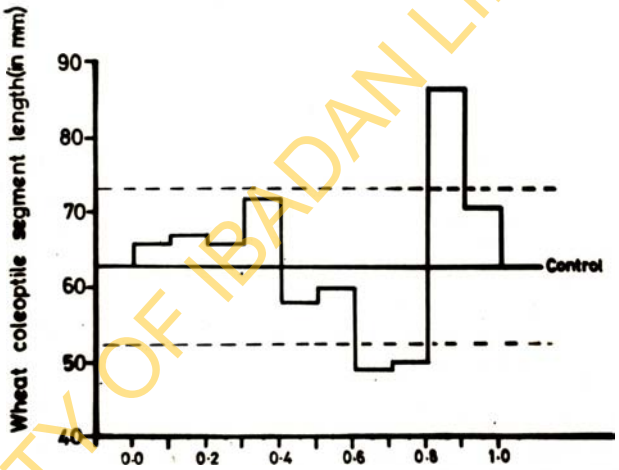


Fig. 22 Wheat coleoptile bioassay on the paper chromatography Rf zones of the AE fraction from 6 days old fruits.

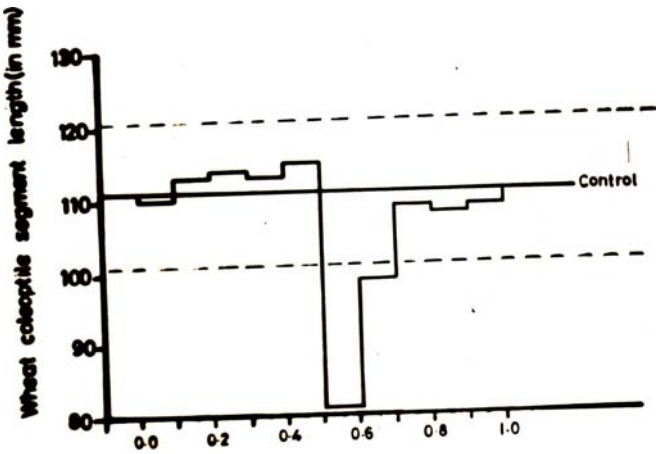


Fig. 23 Wheat coleoptile bioassay on TLC Rf zones of the AE fraction from 2-day old fruits.

Reasonable growth promoting activity was observed at Rf 0.0-0.1 and Rf 0.3-0.4. The activity at Rf 0.0-0.1 was more prominent.

Growth inhibiting activity was observed at Rf 0.5-0.6 and Rf 0.6-0.7. The presence of growth inhibiting activity at Rf 0.5-0.6 and Rf 0.6-0.7 was not unexpected. ABA and its metabolites occurred at these Rf zones in the solvent system that was used. Since these compounds had been identified in the extract by GC-MS, growth inhibitory activity would be expected in these Rf zones.

The compound or compounds that exhibited the growth promoting activity at Rf 0.0-0.1 and 0.3-0.4 were not known. The common acidic natural auxins for example indole-3-acetic acid(IAA), indole-3-propionic acid (IPA), and indole-3-butyric acid(IBA) occurred at higher Rf values (0.5 and above) in the solvent mixture that was used for the TLC. The growth promoting activity that was observed was therefore not considered to be due to any of these.

The MeTMSi derivative of the extract from Rf 0.0-0.1 was analysed on the GC-MS in an attempt to identify the compound or compounds that caused the promotive effect that was observed at Rf 0.0-0.1. Unfortunately none of the compounds that were present could be identified from their mass spectra except the methyl

esters of palmitic and stearic acids. The identity of the growth promoting substance in this Rf zone (0.0-0.1) therefore remained unknown.

(b) Bioassay on Rf zones of paper chromatogram

The PVP purified acidic ethyl acetate extract was subjected to paper chromatography using isopropanol/ammonia/water (10:1:1) as the developing solvent mixture. The results that were obtained in the wheat coleoptile segment bioassay on each of the ten equal Rf zones of the paper chromatogram are shown in Table 5 and the histogram is shown in Figure 22.

TABLE 5

Results obtained in the wheat coleoptile segment bioassay on Rf zones of the paper chromatogram of the acidic ethyl acetate extract from 6-day old fruits

Paper Chromatogram Rf zones	Length of wheat coleoptile segments (mm) 1	2	3	4	5	TOTAL	MEAN
0.0 - 0.1	72	72	56	62	70	332	66 ± 7
0.1 - 0.2	75	68	55	68	70	336	67 ± 7
0.2 - 0.3	66	57	72	72	65	332	66 ± 6
0.3 - 0.4	81	74	72	73	62	362	72 ± 7
0.4 - 0.5	65	53	50	59	64	291	58 ± 7
0.5 - 0.6	62	58	66	55	57	298	60 ± 4
0.6 - 0.7	45	50	50	51	49	245	49 ± 2
0.7 - 0.8	50	50	49	49	51	249	50 ± 1
0.8 - 0.9	90	88	79	85	95	437	87 ± 6
0.9 - 1.0	84	72	64	60	76	356	71 ± 9
CONTROL	63	65	62	63	63	316	63 ± 1

Strong growth inhibiting activity was observed at Rf 0.6 - 0.7 while strong promoting activity was observed at Rf 0.8 - 0.9.

It was thought to be unlikely that any of the common free acidic natural auxins, for example IAA, IPA, and IBA could have been responsible for the growth promoting activity that was observed at Rf 0.8 - 0.9. This was because all of them should occur[169] at lower Rf values of the paper chromatogram in the solvent system that was used for the development.

The approximate Rf values for several auxins on paper chromatograms have been published[93,169]. None of the free acidic natural auxins occurred at about Rf 0.8 - 0.9 when isopropanol/ammonia/water (10:1:1) was used for developing the paper chromatogram. The approximate Rf values[93,169] of some of them were IAA, 0.25 - 0.35; IPA, 0.35 - 0.45, and IBA, 0.45 - 0.55.

The identity of the substance that was responsible for the growth promoting activity that was observed at Rf 0.8 - 0.9 of the paper chromatogram remained unknown.

Analysis of the acidic ethyl acetate (AE) fraction of the extract obtained from two-day old fruits.

The acidic ethyl acetate fraction that was obtained from 2-day old fruits was analysed on the GC-MS as the MeTMSi derivative after purification with PVP. No plant hormone was detected in

the extract by GC-MS, at this stage.

1. Column chromatography of the acidic ethyl acetate fraction that was obtained from 2-day old fruits.

Purification of the acidic ethyl acetate fraction that was obtained from 2-day old fruits was carried out on a charcoal-celite column eluting with a gradient of increasing concentrations of acetone in water. Seventeen 150cm^3 fractions were collected.

(a) TLC analysis of column fractions

Analytical TLC indicated that ABA and its metabolites were probably present in column fractions 4 to 6. The TLC analysis however did not indicate the presence of gibberellins in any of the seventeen column fractions.

(b) Barley half-seed (halo) bioassay of column fractions.

Gibberellin activity was not detected in any of the column fractions.

2. GC-MS analysis of selected column fractions

Selected column fractions (4 to 10) were analysed on the GC-MS as the MeTMSi derivatives after purification with PVP. Fractions 4 to 6 were selected on the basis of the results of the analytical TLC which indicated that ABA and its metabolites were probably present in these fractions. Fractions 7 to 10 were selected just by analogy with the selection made in the case of the extract

from 6-day old fruits.

The results that were obtained in the GC-MS analysis of the selected column fractions of the acidic ethyl acetate extract that was obtained from 2-day old fruits are shown in Table 6 below.

MePA [CLXXVI] and the methyl ester trimethylsilyl ether of 6'-hydroxymethylABA [CLXXXIII] were identified in fraction 4.

MeABA was identified in fractions 5 and 6.

No gibberellin was identified by GC-MS in any of the fractions 4 to 10.

TABLE 6

Plant hormones that were identified in 2-day old fruits of Vigna unguiculata (L.Walp) by GC-MS.

Column fractions	Eluants [% $(CH_3)_2CO$ in H_2O]	Compounds
4	27 - 35	PA and 6'-hydroxymethylABA
5	35 - 40	ABA
6	40 - 45	ABA

3. Wheat coleoptile segment bioassay on the acidic ethyl acetate extract obtained from 2-day old fruits

A portion of the PVP purified acidic ethyl acetate extract that was obtained from 2-day old fruits was subjected to thin-layer chromatography using EtOAc/$CHCl_3$|HOAc (15: 5:1) for the development of the chromatogram. The results that were obtained in the wheat coleoptile segment bioassay that was carried out on each of the ten equal Rf zones of the thin-layer chromatogram are shown in Table 7 below and the histogram is shown in Figure 23.

Strong inhibitory activity was observed at Rf 0.5-0.6 and Rf 0.6-0.7.

No growth promoting activity was observed in any of the Rf zones.

TABLE 7

Results obtained in the wheat coleoptile segment bioassay on TLC Rf zones of acidic ethyl acetate extract from 2-day old fruits.

TLC Rf Zones	Length of wheat coleoptile segments (in mm)											
	1	2	3	4	5	6	7	8	9	10	TOTAL	MEAN
0.0-0.1	110	112	112	103	114	108	107	110	113	112	1101	110±3
0.1-0.2	118	115	105	120	110	113	113	109	115	114	1132	113±4
0.2-0.3	114	110	117	115	114	114	110	120	112	114	1140	114±3
0.3-0.4	125	111	105	101	122	118	120	106	109	112	1129	113±8
0.4-0.5	120	120	115	108	110	116	115	110	119	114	1147	115±4
0.5-0.6	78	80	90	77	80	85	82	76	80	82	810	81±4
0.6-0.7	95	108	98	98	98	97	100	103	97	98	992	99±4
0.7-0.8	118	112	113	104	98	110	110	106	115	104	1090	109±7
0.8-0.9	115	110	112	102	100	108	105	105	114	109	1080	108±5
0.9-1.0	112	115	112	102	102	106	112	107	110	110	1088	109±4
CONTROL	104	110	119	106	116	110	105	115	115	110	1110	111±5

Comparison of the extracts that were obtained from 2-day old and 6-day old fruits

The results that were obtained in the analysis of some of the hormones in the 6-day old fruits and the 2-day old fruits clearly showed a disparity in the hormonal contents of these two groups of fruits.

While several gibberellins (Table 3, page 162) were identified in the 6-day old fruits, no gibberellin (Table 6, page 170) could be identified in the 2-day old fruits.

Growth promoting activity was observed in the auxin bioassay (wheat coleoptile segment bioassay) carried out on the extract from 6-day old fruits (Tables 4 and 5, pages 164 and 167 and Figs. 21 and 22, page 164a). No growth promoting activity was however observed when the same test was carried out on the extract from 2-day old fruits (Table 7, page 172 and Figure 23, page 164a). Thus while auxins could be detected in the 6-day old fruits, they could not be detected in the 2-day old fruits.

It therefore seemed that while gibberellins and auxins (growth promoting hormones) were present in the 6-day old fruits, they were absent in the 2-day old fruits. If these growth promoters were present in the 2-day old fruits at all, their concentrations must have been so low that they could not be detected.

As mentioned earlier, under the Introduction (page 15), the plant hormones constitute the major internal factor governing abscission in plants. The growth promoters (auxins, gibberellins and cytokinins) promote the growth of plant organs and therefore normally inhibit the abscission of these organs. The growth inhibitors on the other hand, inhibit the development of plant organs and so normally promote the abscission of these organs. Abscisic acid and its metabolites therefore accelerate the abscission of plant organs, such as leaves, flower buds, flowers and fruits.

Another internal factor which regulates the abscission of plant organs is competition for available nutrients. This factor is also related to the hormonal content of the plant organs. This is because one major role of the growth promoters in relation to abscission is in the mobilization of nutrients. Thus plant organs which have high concentrations of the growth promoters deprive organs which have low concentrations of these growth promoting hormones of nutrients. The organs that are deprived of nutrients will then starve and abscise.

In the light of what has been written above about the role of plant hormones in relation to abscission, the results that were obtained in the analysis of the hormonal content of the two

groups of cowpea fruits could be used to explain the abscission problem in cowpea.

The results that were obtained in the analysis of some of the growth promoting hormones (GAs and auxins) in the two groups of cowpea fruits clearly indicated the presence of these growth promoters in the 6-day old fruits. On the other hand, these growth promoters were not detected in the 2-day old fruits. It could therefore be expected that the growth promoters in the older (6-day old) fruits of the lowest raceme would lead to the mobilization of available nutrients towards these fruits. The younger fruits of the upper racemes which were deficient in the growth promoters (and therefore deficient in nutrient "mobilizers") would then starve and abscise.

The interpretation of the results given above would then explain the observation of Ojehomon[1,2,4] that the abscission of the immature fruits and flowers of the upper racemes of the cowpea inflorescence were associated with the presence of maturing fruits at the lowest raceme. It would also explain his observation[2] that the lateral flowers of each raceme were capable of growing to mature fruits if the older fruits below were removed.

The interpretation would also agree with the results of the investigations by Adedipe and Ormrod[6] and Adedipe et al[7] in which they showed that competition for nutrients was an important factor

in the abscission problem in cowpea.

Apart from the mobilization of nutrient, the growth promoting hormones can also have direct effect on abscission. The growth of plants and plant organs is partly controlled by an interaction between the growth promoting hormones and the growth inhibiting hormones. If the balance of growth regulators is in favour of the growth promoters, the growth of the organ will progress. If however the growth inhibitors are favoured relative to promoters, the growth of the plant organ is arrested.

The 6-day old fruits had both growth promoting hormones (GAs and auxins) and growth inhibitors (ABA and its metabolites). The 2-day old fruits however had growth inhibitors but no detectable amount of the growth promoters. Thus the older fruits would have an adequate concentration of the growth promoters to counteract the effect of the growth inhibitors. The growth of these fruits would thus progress to maturity. In contrast, the 2-day old fruits (from the upper racemes) had no detectable amount of growth promoters (GAs and auxins) to counteract the inhibitory effect of ABA and some of its metabolites that were identified in these fruits. The growth of these fruits would therefore be arrested and they would finally abscise.

The inducing influence which the maturing fruits at the first raceme had on the abscission of the younger upper fruits could then be due to the abscising influence of hormones which were translocated in the acropetal (apical) direction from the maturing basal fruits.

Both growth promoters and inhibitors accelerate[8] abscission when applied proximally to the abscission zone. Thus plant hormones (both growth promoters and inhibitors) entering the peducle from the maturing fruits of the lowest raceme and translocated in the acropetal (apical) direction would reach the younger, upper fruits from the proximal end. They would therefore accelerate the abscission of these younger, upper fruits. This would thus agree with Ojehomon[2]'s second hypothesis that the abscission of the immature fruits were stimulated by substances produced in the lower, older fruits.

Van Steveninck[170,171] had postulated a similar hypothesis to explain the abscising influence of maturing fruits, at the base of yellow lupin (Lupinus luteus) inflorescence on the abscission of younger, upper fruits. (Yellow lupin exhibits an abscission problem quite similar to that of cowpea).

Wheat coleoptile straight growth test on the extract which he obtained from 7-day old fruits indicated the presence of growth

promoter/s and inhibitor/s in the extract.

The main component of the inhibiting fraction was later identified by Cornforth et al[172a] to be abscisic acid.

Analysis of the acidic ethyl acetate (AE) fraction of the extract obtained from fruits over 6 days old

The acidic ethyl acetate extract that was obtained from fruits that were over six days old was also analysed. This was in order to compare the hormonal content with the hormonal contents of the 6-day old and the 2-day old fruits.

The PVP purified extract was analysed on the GC-MS as the MeTMSi derivative. The gas chromatogram had very many peaks indicating the presence of many compounds most of which could not be identified from their mass spectra.

The mass spectrum of one of the peaks however indicated that it was a mixture of MeA_6TMSi [CLXXIV] and $MeA_{17}TMSi$ [CLXXV]. All the prominent ions in the mass spectra of MeA_6TMSi and $MeA_{17}TMSi$ as discussed earlier in this chapter (page 126) were present in the mass spectrum of the peak.

The extract was further purified on a charcoal-celite column eluted with a gradient of increasing concentrations of acetone in water.

GC-MS analysis of the MeTMSi derivatives of some of the PVP purified column fractions afforded the identification of MePA [CLXXVI], MeDPATMSi [CLXXXII], iso-MeDPATMSi, MeABA [CLXCIV], Me6'-hydroxymethylABATMSi [CLXXXIII], MeA_6TMSi [CLXXIV], MeA_8TMSi [CLXCI] and possibly MeA_{20}TMSi [CLXCIX].

The hormonal content of the fruits that were over 6 days old was thus basically similar to the hormonal content of the 6-day old fruits. More gibberellins were however identified in the extract from 6-day old fruits than from the extract from fruits that were over six days old. This was not surprising since the gibberellin content of plant organs generally decrease[172b] as the organs get older.

Analysis of the extracts obtained separately from the seeds and from the fruit wall of 6-day old fruits

One problem that was constantly encountered during the GC-MS analysis of the extracts that were obtained from the 6-day and 2-day old fruits was the presence of a lot of impurities in these extracts. These impurities interfered seriously with the analysis. The various methods that were used to purify the extracts did not succeed in removing a substantial amount of the impurities.

An attempt was therefore made to find out whether the impurities were from the seeds or from the fruit wall. Most of the

plant hormones would normally be expected to be in the seeds.

The seeds of some 6-day old fruits were separated from the fruit walls and separate extracts were then obtained from the seeds and the fruit walls.

Without further purification, the acidic ethyl acetate fraction of each extract was analysed on the GC-MS as the MeTMSi derivative.

The GC-MS analysis of the derivatized crude ethyl acetate fraction obtained from the seeds afforded the identification of MeA_1TMSi [CLXCVI], MeA_4TMSi [CC], MeA_5TMSi [CLXCVIII], MeA_6TMSi [CLXXIV], MeA_8TMSI [CLXCI], $MeA_{17}TMSi$ [CLXXV], $MeA_{20}TMSi$ [CLXCIX], MeTMSi derivative of gibberellin Y [CLXCV] and possibly $MeA_{19}TMSi$ [CCI] in the extract. Although the mass spectra obtained for many of these compounds were contaminated with impurities, they were considered good enough for the identifications to be made.

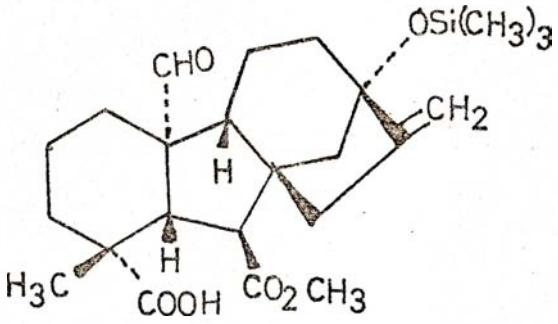
Only MeA_6TMSi could be identified in the derivatized (MeTMSi) crude ethyl acetate fraction that was obtained from the fruit walls. All the other compounds could not be identified. Most of them were thought to be phenolic impurities.

The result that was obtained for the GC-MS analysis of the crude acidic ethyl acetate fraction obtained from 6-day old seeds was very interesting in that many gibberellins were identified. This result could be compared with the result that was obtained for the

GC-MS analysis of the acidic ethyl acetate fraction obtained from 6-day old fruits, after a one step purification with PVP (page 126). Only MeA_6TMSi and $MeA_{17}TMSi$ could be easily identified from the extract from 6-day old fruits after the one step purification with PVP. The other hormones were identified only after further purification of the extract by column chromatography. This thus showed that the extract from the seeds contained less amount of impurities than the extract from the fruits. Therefore the major part of the impurities that were encountered in the analysis of the extracts obtained from fruits were from the fruit walls and not from the seeds. This conclusion was further confirmed by the result that was obtained above for the GC-MS analysis of the extract from the fruit walls.

It therefore appeared advisable that any further investigations of the hormonal content of cowpea fruits should be concentrated on the seeds.

The acidic ethyl acetate extract obtained from some 6-day old fruits which were extracted in a soxhlet extractor with 80% aqueous methanol was used for some preliminary biological assays.



Me A_{19}TMSi

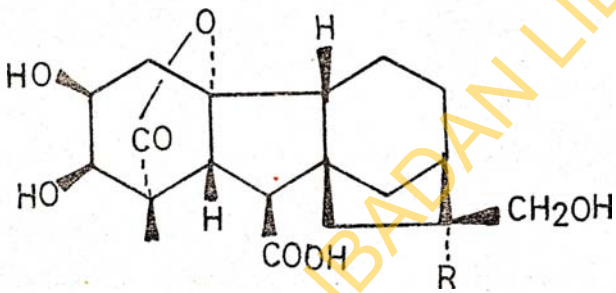
(C C I)

Partial synthesis of the structures that were tentatively assigned to gibberellins X and Y

As stated earlier in this chapter, two new compounds were detected in the extract that was obtained from 6-day old fruits. They were believed to be new gibberellins from the evidence obtained from the mass spectra of their MeTMSi derivatives. They were tentatively referred to as gibberellins X and Y.

On the basis of the mass spectral evidence (page 143), the structure of gibberellin X was thought to be 16α, 17-dihydroxy derivative of gibberellin A_{34} [CCIIa]. The stereochemistry of the 2,3-hydroxyl groups was not certain, but 2β, 3β was thought to be

the most probable by analogy with the structures of the known C_{19}-gibberellins. The stereochemistry of the 16-hydroxyl group was also not certain. The α-configuration was however thought to be more likely by comparison with the structures of the known gibberellins (Fig. 1).



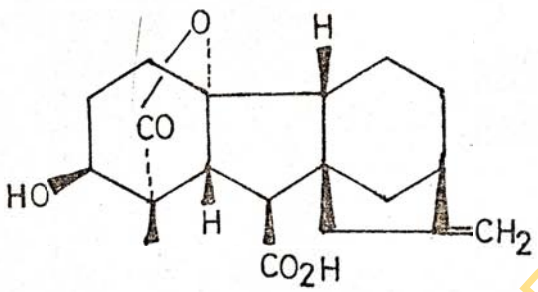
Gibberellin X (CCIIa) R = OH

Gibberellin Y (CCIIb) R = H

The structure of gibberellin Y was also thought to be probably 17-hydroxy derivative of gibberellin A_{34} [CCIIb]. Although the configurations at C-2, C-3 and C-16 were not certain, the most likely configuration at these positions were thought to be as shown in CCIIb.

In order to determine whether the tentatively assigned structures [CCIIa] and [CCIIb] for the two new gibberellins were correct, it was decided to carry out their partial synthesis.

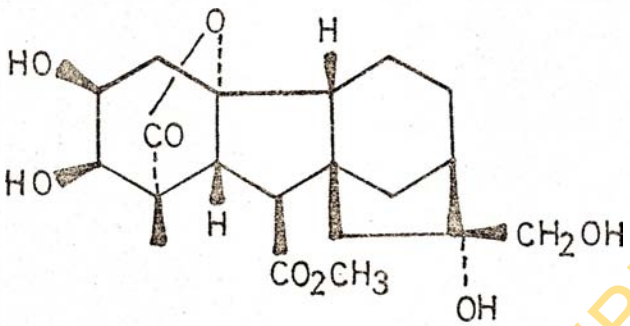
Gibberellin A_4, a relatively abundant gibberellin was used as the starting material for the partial synthesis.



GA_4
(XIV)

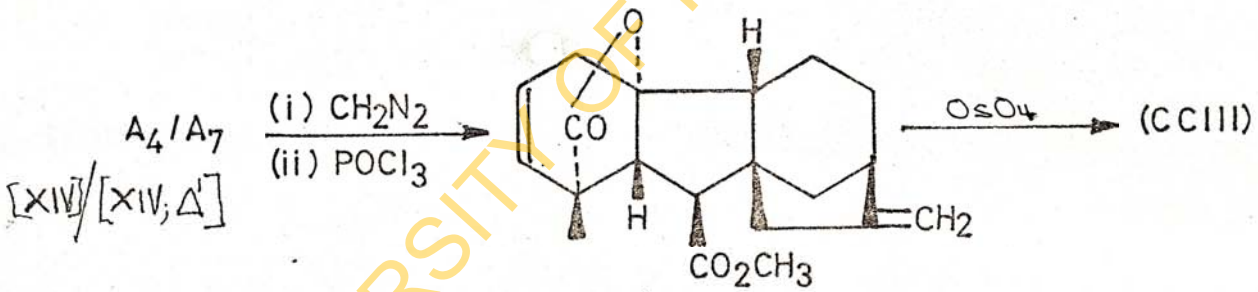
1. Partial synthesis of the structure that was tentatively assigned to gibberellin X and the 16-epimer

In order to find out whether the structure [CCIIa] that was tentatively assigned to gibberellin X was correct, it was decided to synthesize the methyl ester of 16α, 17-dihydroxyMeGA$_{34}$ [CCIII] from gibberellin A_4 [XIV] as shown in scheme 37 below.



(CCIII)

SCHEME 37



(CCIV)

A mixture of GA_4 and GA_7 (70% GA_4) was methylated with ethereal diazomethane. The crude methyl ester without further purification was dehydrated[29,31,43] with phosphorus oxychloride in pyridine to give 2,3-dehydroGA_9 methyl ester [CCIV].

Alcohols are readily converted into olefins by phosphorus oxychloride in pyridine. The reaction is believed to proceed via an intermediate dichlorophosphate ester. The abstraction of a trans-vicinal proton by the base (pyridine) and the concerted loss of the dichlorophosphate group result in the olefin[173].

The 2,3-olefinic protons of the olefin [CCIV] were observed as a multiplet centered at δ5.75 in the n.m.r. spectrum of the compound in deuterochloroform. The 5-H and the 6-H appeared as doublets centered at δ2.65 and δ2.83 respectively. The C-17 methylene protons were observed as two broad peaks at δ4.99 and δ4.88. The C-18 methyl protons which were deshielded by the effect of the double bond in the A ring appeared as a singlet at δ1.23. These chemical shifts were consistent with those reported in literature[32] for the compound.

The mass spectrum had a molecular ion at m/e 328. There were some other prominent ions at m/e 297 (M^+-CH_3O), m/e 284 (M^+-CO_2), m/e 225 (M^+-CO_2, CH_3COO) and m/e 225 (base peak, M^+-CO_2, CH_3COOH).

The methyl 2,3-dehydroGA$_9$ was then hydroxylated by treatment with osmium tetroxide in pyridine giving the 16α, 17-dihydroxyMeGA$_{34}$ [CCIII].

The treatment of olefins with osmium tetroxide to give 1,2 cis diols via an intermediate osmate ester is well described in literature[174]. The osmium tetroxide is believed to react with the olefin to form a cyclic osmate ester. The osmate ester can then be cleaved with a series of reagents[175] such as strong aqueous base and mannitol, hydrogen sulphide, refluxing aqueous alcoholic sodium sulphite or bisulphite, and aqueous pyridine and sodium bisulphite.

The β orientation of the 2,3-hydroxyl groups was assumed on the basis that the attack of the olefin [CCIV] by the osmium tetroxide was more likely from the less hindered β face of ring A. Osmium tetroxide hydroxylation of 2,3-double bond of gibberellins had been reported[43,56] to give the 2β, 3β-diol system.

The n.m.r. spectrum of the product [CCIII] in C_5D_5N clearly showed that the 2,3-hydroxyl groups had the β configuration. This was because of the downfield shift of the 5-H signal which was observed as a doublet centred at δ3.78. The downfield shift was due to the deshielding effect of the 1,3-diaxial interaction with the 3β-hydroxyl group. This effect had been reported[32] to be very useful in determining the configuration of the 3-hydroxyl group. The 2-H and the 3-H were observed as a multiplet centred at δ4.32 and a doublet centred at δ4.13 respectively. The 6-H appeared

as a doublet at δ3.05. The C-17 methylene protons appeared as a doublet at δ3.97. The n.m.r. data was therefore consistent with structure [CCIII].

The 16-hydroxyl group was assumed to have the α configuration on the basis that the bulky osmium tetroxide would attack the exocyclic double bond from the less hindred α face of ring D. Osmylation reactions are known to proceed from the less hindered side of the molecule[176].

The mass spectrum (Fig. 24) of the TMSi ether of [CCIII] had a molecular ion at m/e 684. There was a prominent M^+-103 (M^+-CH_2OTMSi) ion which was the base peak. Also present were the ions at m/e 73, m/e 75 and M^+-15 associated with the TMSi ether group. There were the prominent ions at m/e 129 (indicative of 3-hydroxylation), m/e 147 and m/e 217 (associated with the 2,3-diol system).

This mass spectrum (Fig. 24) of the TMSi ether of 16α, 17-dihydroxyMeA$_{34}$ was very similar to the mass spectrum (Fig. 16, page 143a) of the MeTMSi ether of gibberellin X. There were however a few ions in the mass spectrum of the MeTMSi of gibberellin X which were absent in the mass spectrum of the TMSi ether of 16α, 17-dihydroxy-MeA$_{34}$. These ions, especially the prominent one at m/e 204 could be due to impurities. That these ions were due to impurities was supported by the fact that the same ions were prominent in the mass spectra of the three preceeding components (P46, P47 and P48) shown

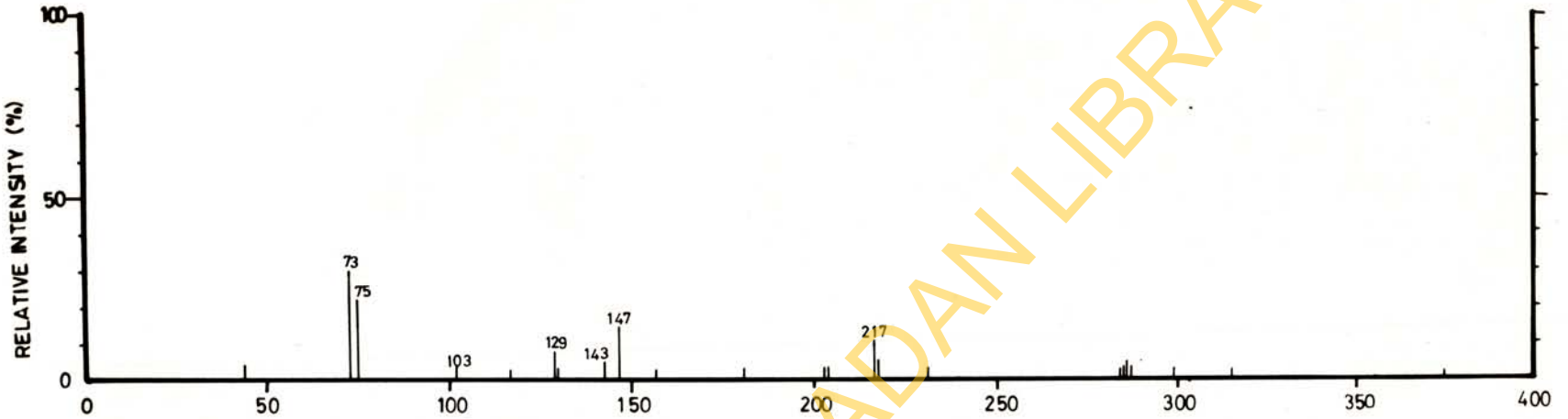


Fig. 24 Mass spectrum of Me 16α-17-dihydroxyA34 TMSi

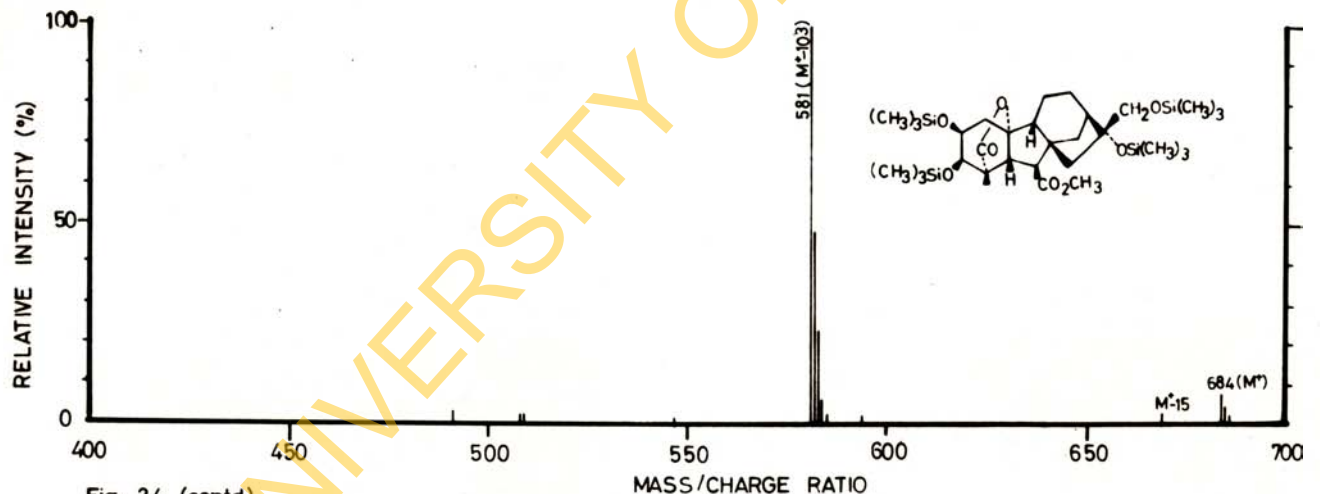


Fig. 24 (contd.)

on the gas chromatogram (Fig. 8 page 130a).

The retention time of the MeTMSi derivative of gibberellin X was however different from the retention time of the TMSi ether of CCIII. It thus followed that the two compounds were not identical. The fact that their mass spectra were similar however showed that they were closely related structurally.

It was thought that the natural compound might be the epimer of the synthetic compound. The epimerization was thought to be more likely at C-16.

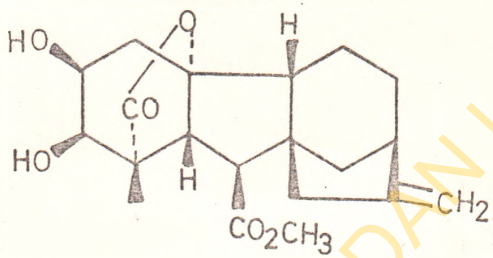
It was therefore decided to prepare the 16-epimer of CCIII in order to check whether the TMSi ether of this would have the same retention time with the MeTMSi ether of gibberellin X.

The preparation of the 16-epimer of CCIII would involve the hydroxylation of the exocyclic double bond of $MeGA_{34}$ [CCV] from the more hindered face.

An important method for the preparation of cis diols involving the introduction of the cis hydroxyl groups on the more hindered side of the molecule is the Woodward[177] reaction. This is the reaction of olefins with iodine and silver acetate in wet acetic acid. Although there had been no report of the use of this reaction in gibberellin chemistry, it had been used[178] in the total synthesis of some steroids. The Woodward reaction would thus give a cis

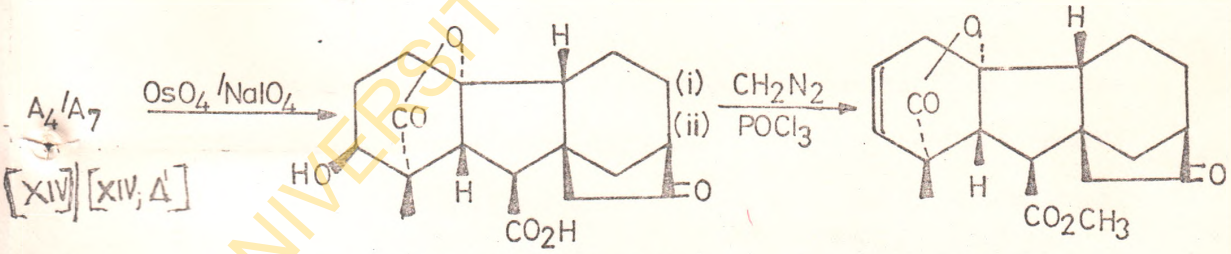
glycol with opposite stereochemistry to that derived from osmium tetroxide.

The projected plan for the synthesis of the 16-epimer of CCIII is shown in scheme 38 below. The first part of the scheme consisted of the synthesis of MeA_{34} [CCV] from GA_4 [XIV] following the method used by Beeley and MacMillan[43].



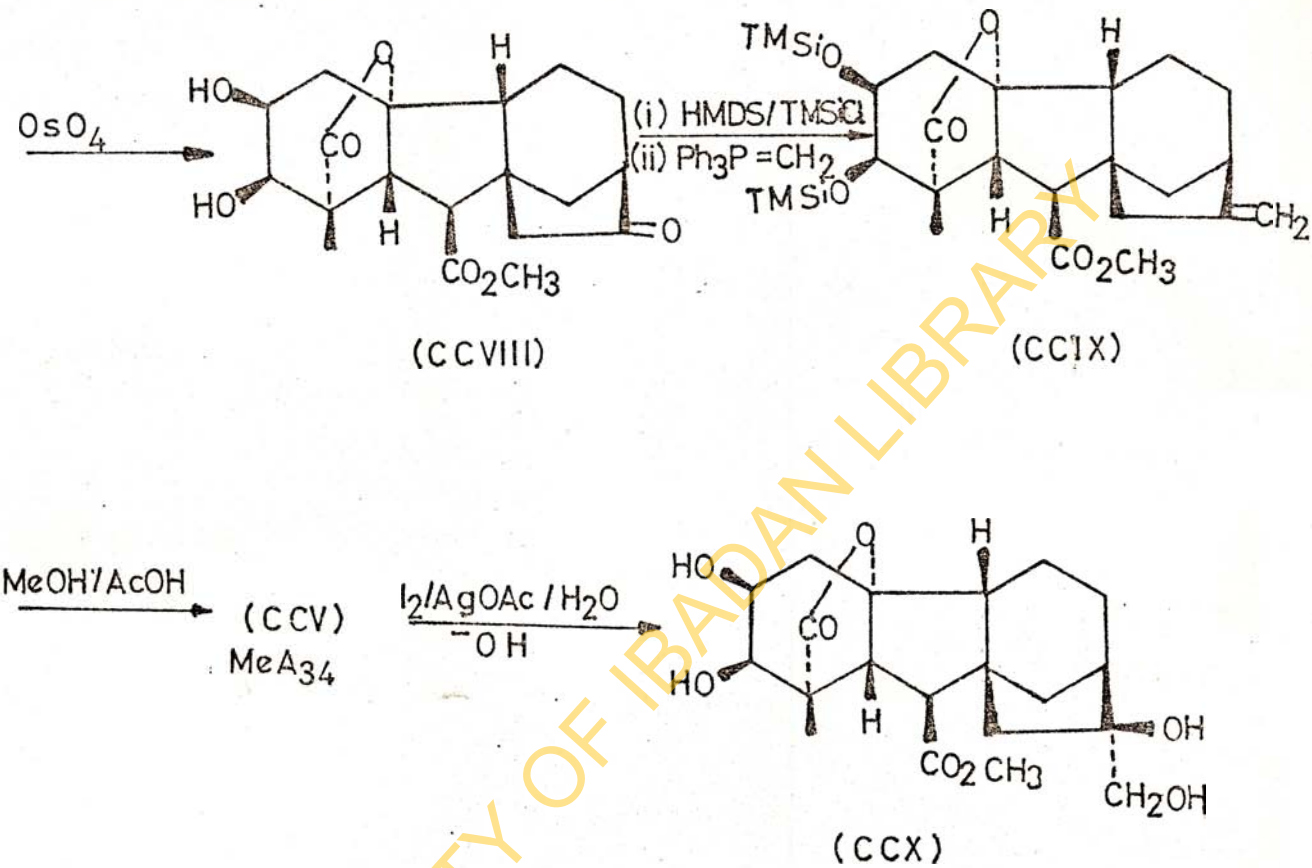
MeA_{34} (CCV)

SCHEME 38



GA_4 norketone (CCVI) (CCVII)

Scheme 38 contd.



A mixture of GA_4 and GA_7 (70%GA_4) was treated[43] in pyridine with sodium metaperiodate and a small amount of osmium tetroxide. GA_4 norketone [CCVI] was obtained in a good yield. The melting point (120-136°) was in close agreement with the reported[179] m.p. (120-140°) for GA_4 norketone. The mass spectrum of the MeTMSi derivative of the GA_4 norketone (M^+ 420) gave the fragmentation pattern expected for the MeTMSi derivative of GA_4 norketone.

This oxidative cleavage of the 16,17-exocyclic double bond was effected in order to prevent its being attacked by OsO_4 later in the scheme during the hydroxylation of the 2,3-positions. Combinations of periodate with osmium tetroxide or permanganate are usually used for this type of cleavage. This is because the osmium tetroxide or the permanganate converts the olefin to 1,2 glycol which is then cleaved by the periodate.

The norketone was methylated with ethereal diazomethane and the resulting crude methyl ester was dehydrated[43] using phosphorus oxychloride in pyridine. The resulting olefin, methyl 2,3-dehydroGA_9 norketone [CCVII] was obtained in about 60% yield.

In the n.m.r. spectrum of the olefin [CCVII] in deuterochloroform ($CDCl_3$), the olefinic protons were observed as a 2H-multiplet at δ5.6 to δ5.9. The 5-H and the 6-H protons were observed as two doublets centred at δ2.5 (J10Hz) and δ2.68 (J10Hz) respectively. The C-18 methyl protons and the three methoxy protons appeared as two 3H-singlets at δ1.23 and δ3.73 respectively. Both this n.m.r. spectrum of the olefin and the melting point (158 - 160°) were in close agreement with those reported[73] in literature.

Treatment[43] of the olefin [CCVII] with osmium tetroxide in pyridine gave methyl GA_{34} norketone [CCVIII].

The fragmentation pattern of the mass spectrum of the TMSi derivative of the methyl GA_{34} norketone that was obtained was in agreement with what was expected. It had a molecular ion (base peak) at m/e 508. There were prominent ions at m/e 147 and m/e 217 (indicative of 2,3 diol system). There were also other important ions at m/e 73, 75, 129, 477 (M^+-31) and 493 (M^+-15).

As stated earlier, the β, β configuration of the 2,3-hydroxyl groups of the norketone [CCVIII] was assumed on the basis of steric considerations. Since hydroxylation of olefins with osmium tetroxide occur from the less hindered side, the attack of the olefin [CCVII] by the bulky osmium tetroxide would be expected to be from the less hindered β face of ring A.

The 2,3-vicinal diol system of methyl GA_{34} norketone [CCVIII] were then protected[43] as the TMSi ethers. This was achieved by silylating the diol with a mixture of hexamethyldisilazane, trimethylsilyl chloride and pyridine (2: 1: 2). The diol system was protected as the TMSi ethers because it was reported[180] that attempted Wittig[181] reaction on the unprotected diol norketone [CCVIII] did not give satisfactory results. This was probably due to the attack of the ylid on the unprotected hydroxyl groups of the diol norketone.

The protected diol norketone was then treated[43] with methylenetriphenylphosphorane giving the MeTMSi derivative of GA_{34} [CCIX]. The conversion of aldehydes and ketones to olefins by means of the Wittig reaction[181] is well known in chemistry.

The hydrolysis of the MeTMSi derivative of GA_{34} with a 4:1 mixture of methanol/acetic acid gave MeA_{34} [CCV] in low yield.

In the n.m.r. spectrum of MeA_{34} in $CDCl_3$, the exocyclic methylene protons were observed at δ4.96. The 5-H and the 6-H protons appeared as two doublets at δ3.19 and δ2.70 respectively. The downfield shift of the 5-H signal was due to the deshielding[32] by a 3β-hydroxyl group. This confirmed the 2β, 3β-configuration of the 2,3-diol system.

The mass spectrum of the TMSi derivative of the $MeGA_{34}$ was consistent with what was expected. It showed a molecular ion at m/e 506 (base peak). There were prominent ions at m/e 147 and 217 indicative of the 2,3-diol system.

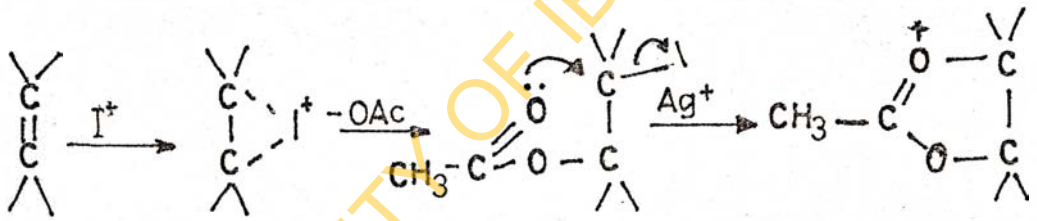
The next stage in the synthesis was the hydroxylation of the exocyclic double bond from the more hindered side of the molecule.

As stated earlier, the Woodward reaction[177] should lead to the introduction of <u>cis</u> hydroxyl groups from the more hindered side of ring D of the gibberellin molecule.

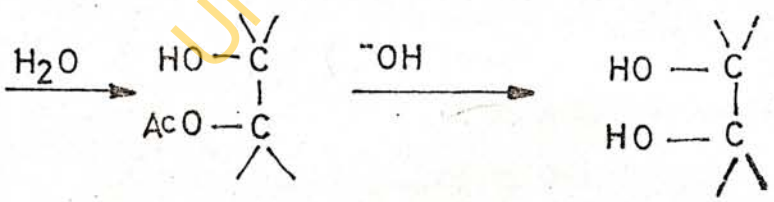
The mechanism of the Woodward reaction is believed to be as shown in scheme 39 below.

An olefin [CCXI] reacts with I^+ forming an intermediate cyclic iodonium ion [CCXII]. It is believed that the I^+ is derived from acetylhypoiodide, CH_3COOI, formed from silver acetate and iodine. The iodonium ion is opened by an acetate group to give a trans-acetoxy iodide [CCXIII]. The acetate group of the acetoxy iodide displaces the adjacent iodide ion giving a cyclic acetoxonium ion [CCXIV]. Water (from wet acetic acid) then adds to the acetoxonium ion, giving a cis hydroxy acetate [CCXV]. Hydrolysis of this gives the cis diol [CCXVI].

SCHEME 39



(CCXI) (CCXII) (CCXIII) (CCXIV)



(CCXV) (CCXVI)

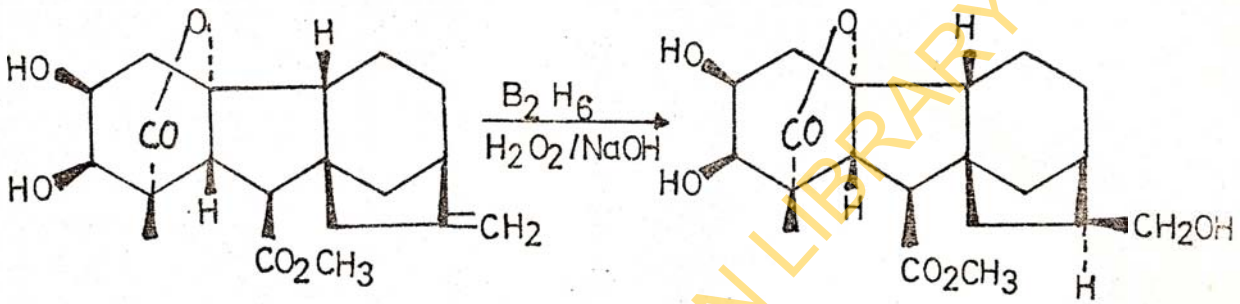
The Woodward hydroxylation was carried out on MeA_{34}. The final product was silylated and analysed on the GC-MS. The analysis showed that there were many compounds in the final product of the Woodward reaction. Three of the compounds however had the expected molecular ion at m/e 684. It was therefore likely that one of these was the TMSi ether of CCX. One of these three had the same retention time with the MeTMSi ether of gibberellin X. Each of the three components however had some prominent ions in its mass spectrum which were not present in the mass spectrum of the MeTMSi of gibberellin X.

2. Partial synthesis of the tentatively assigned structure of gibberellin Y and the 16-epimer

Since gibberellin Y was thought to be probably 17-hydroxyGA_{34} [CCIIb], it was decided to synthesize the methyl ester of 17-hydroxyGA_{34} [CCXVII] in order to check whether the tentatively assigned structure was correct.

The 17-hydroxyGA_{34} methyl ester [CCXVII] was synthesized from MeA_{34} as shown in scheme 40 below.

SCHEME 40



(CCV) (CCXVII)

Hydroboration[182,183] is a versatile method for the anti-Markovnikov hydration of double bonds. It involves a cis anti-Markovnikov addition from the less hindered side of the double bond. It was therefore thought that hydroboration should provide a nice method for preparing the methyl ester of the structure that was tentatively assigned to gibberellin Y [CCXVII] from MeA_{34}.

MeA_{34} in dry tetrahydrofuran (THF) was treated with a solution of diborane (B_2H_6) in THF, under nitrogen gas. The resulting organoborane was oxidised with hydrogen peroxide in the presence of aqueous NaOH. The gummy product was analysed on the GC-MS as the

TMSi ether.

The GC-MS analysis of the product revealed that it was a single compound. The general fragmentation pattern of the mass spectrum (Fig. 25) of the TMSi of this compound was consistent with what would be expected from the TMSi of [CCXVII]. It had a molecular ion (base peak) at m/e 596. There was a prominent ion at m/e 103 indicating the presence of the $-CH_{20}TMSi$ group. There was an ion at m/e 129 indicating the presence of 3-hydroxyl group. Prominent ions at m/e 147 and 217 indicated the presence of the 2,3-diol system. There was a significant ion at m/e 506 (M^+-90) which was probably due to the loss of a TMSiOH group from the molecular ion. The first half of the mass spectrum was very similar to the mass spectrum of $MeA_{34}TMSi$ except that the ion at m/e 103 was much more prominent.

Although the mass spectrum (Fig. 25) of the TMSi derivative of the hydroboration product was similar to the mass spectrum (Fig. 19, page 149a) of the MeTMSi derivative of gibberellin Y (the natural compound), there were some significant differences. Moreover their retention times on GLC were not the same, although they were close.

It therefore followed that the structure [CCIIb] that was tentatively assigned to gibberellin Y was not correct. It was however thought that the natural compound might be the epimer of

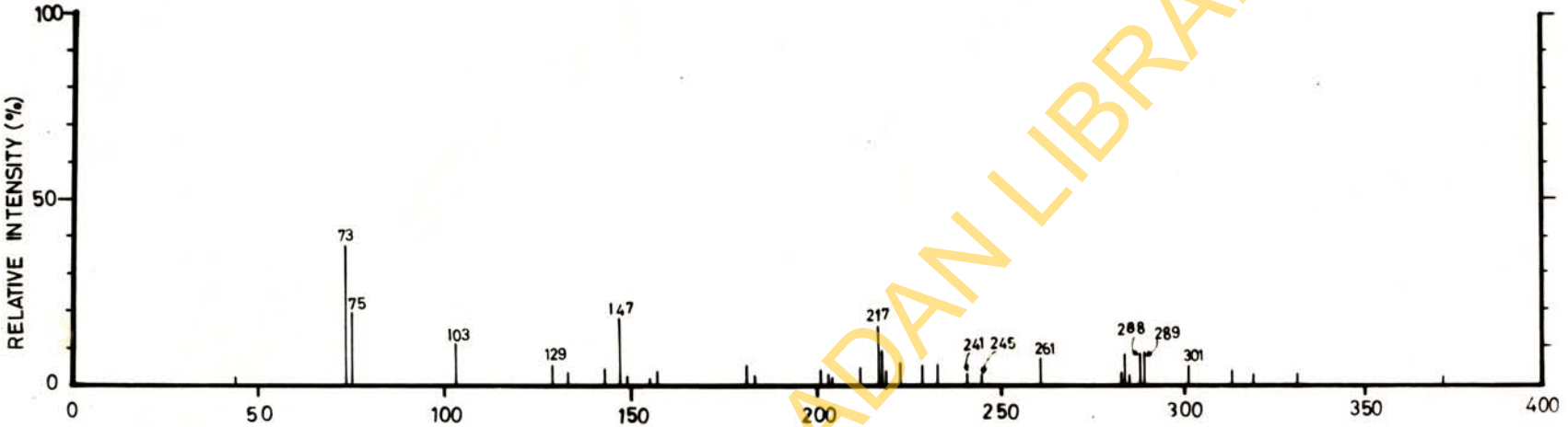


Fig. 25. Mass spectrum of Me17-hydroxy A_{34}TMSi

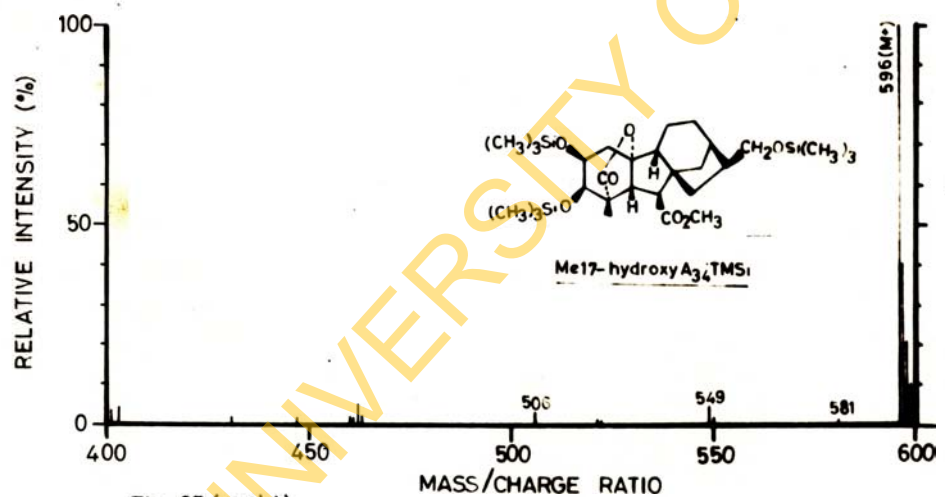


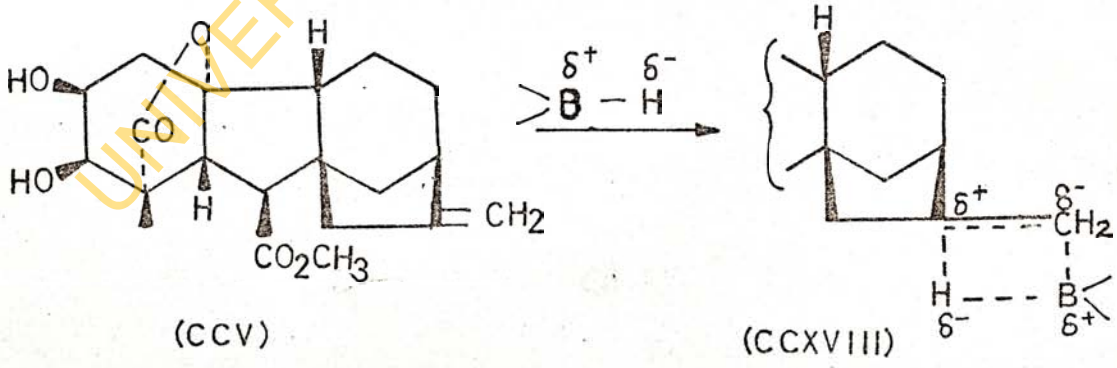
Fig. 25 (contd.)

the synthetic compound. This was because of the similarity of the mass spectra of their MeTMSi derivatives. Also although their retention times were not the same, they were quite close.

The epimerisation was thought to be more likely at C-16. The stereochemistry of the hydroboration product [CCXVII] at C-16 had been assumed to be as shown on the basis that the reagent would attack the double bond from the less hindered α face of ring D.

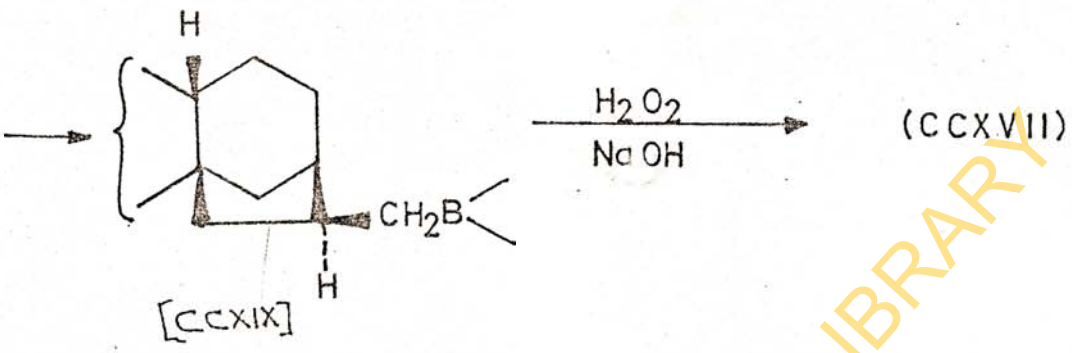
The mechanism of the hydroboration reaction could be envisaged to have taken place as shown in scheme 41 below leading to the formation of CCXVII.

SCHEME 41



(CCV) (CCXVIII)

Scheme 41 contd

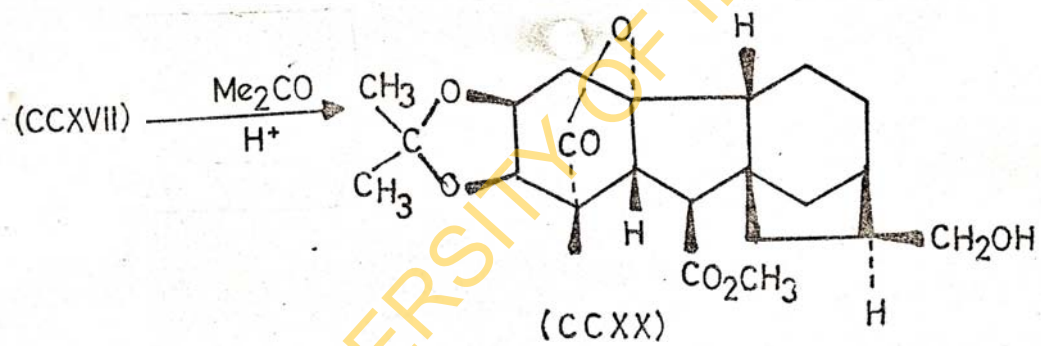


The attack on the exocyclic double bond of MeA_{34} [CCV] would be initiated by the polarisation of the boron-hydrogen bond. The attack would proceed on the less hindered α-face leading to the formation of the alkyl borane [CCXIX], through a four-centred transition state [CCXVIII]. Oxidation of the alkyl borane [CCXIX] with hydrogen peroxide in the presence of alkali would give the alcohol [CCXVII]. This mechanism for the hydroboration reaction clearly indicated that the stereochemistry at C-16 of the hydroboration product should be as shown in [CCXVII]. That is the orientation of the 17-CH_2OH group should be β.

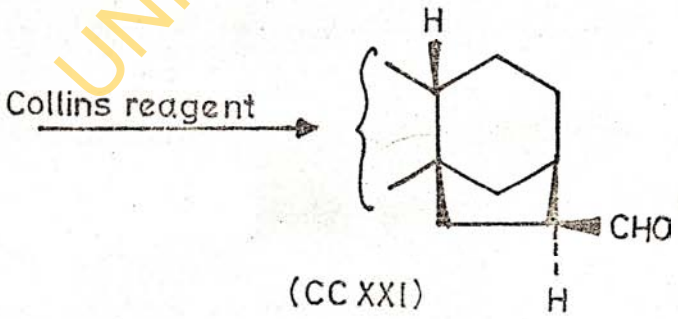
It was therefore decided to prepare the 16-epimer of CCXVII. The orientation of the 17-CH_2OH group would be α in this compound. The 16-epimer of CCXVII was prepared as shown in shceme 42.

Scheme 42 involved the protection of the 2,3 diol system of CCXVII as the acetonide [CCXX] followed by the oxidation of the 17-hydroxyl to give the aldehyde [CCXXI]. Hopefully the aldehyde could then be epimerised with base. Reduction of the epimerised aldehyde [CCXXII] and the removal of the protecting group of the 2,3-diol would give the 16-epimer [CCXXIII] of CCXVII. These reactions were done on small scale and were therefore followed with GC-MS analysis.

SCHEME 42

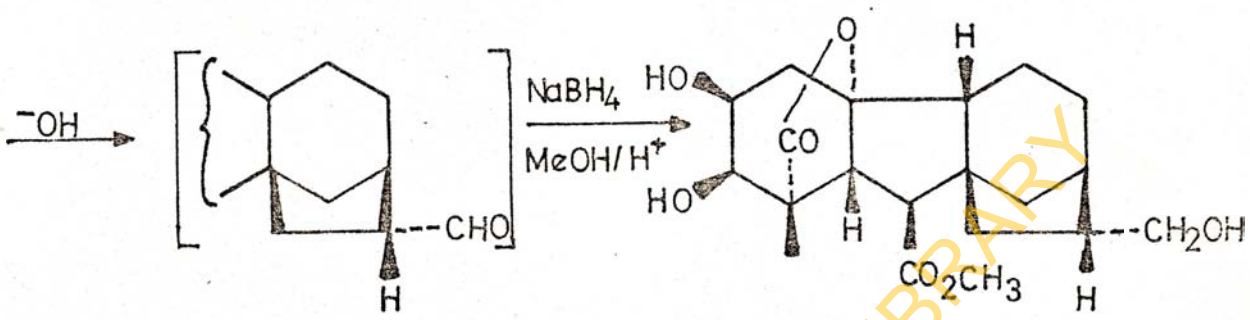


(CCXX)



(CCXXI)

Scheme 42 contd.



(CCXXII) (CCXXIII)

The acetonide [CCXX] was prepared by dissolving the triol [CCXVII] in dry acetone containing about 0.001% conc.H_2SO_4 at room temperature for about 24 hours. The GC-MS analysis of the product indicated the presence of the acetonide as the main product.

The mass spectrum had a molecular ion at m/e 420. There were some other prominent ions at m/e 405 (M^+-CH_3), m/e 403 (M^+-OH), m/e 402 (M^+-H_2O), m/e 388 (M^+-CH_3OH), m/e 387 (M^+-H_2O,CH_3), m/e 344 ($M^+-CH_3COCH_3$, H_2O), m/e 312 ($M^+-CH_3COCH_3$, CH_3OH, H_2O), m/e 284 ($M^+-CH_3COCH_3$, CH_3COOH, H_2O), m/e 241 ($M^+-CH_3COCH_3$, CH_3COO, CO_2,H_2O) m/e 239 ($M^+-CH_3COCH_3$, CH_3COO, HCOOH, H_2O), m/e 223 (base peak) ($M^+-CH_3COCH_3$, CH_3COOH, HCOOH, CH_3, H_2O) and m/e 43 (CH_3CO^+).

The acetonide linkage is widely used as a protecting group for 1,2-cis diols[184]. It is stable to alkaline conditions[185] but easily cleaved with acid[186].

As the acetonide linkage (protecting group) is labile to acid, the oxidation of the 17-hydroxyl group had to be effected with nonacidic reagent. A suitable nonacidic reagent which has been used extensively to oxidise primary alcohols to aldehydes is dipyridine-chromium[VI] oxide complex in dichloromethane (Collins reagent)[187]. The acetonide linkage has been reported to be stable to Collins reagent[188].

The alcohol [CCXI] was oxidised with the complex prepared in situ following the method of Ratcliffe[189]. Analysis of the product on GC-MS indicated the presence of the aldehyde [CCXXI] as the major product.

The mass spectrum of the aldehyde had a molecular ion at m/e 418. There were prominent ions at m/e 403 (M^+-CH_3), 300 (M^+-CH_3, CH_3CO, CH_3COOH), 257 (M^+-CO_2, CH_3COOH, CH_3COCH_3), 239 (base peak) (M^+-H_2O, CO_2, CH_3COOH, CH_3COCH_3) and m/e 43 (CH_3CO^+).

The next stage was the epimerization of the aldehyde (CHO) group. It was decided to do this in a basic medium since the acetonide protecting group would be stable to the basic condition. Epimerization of suitable substituents on the gibberellin skeleton

have been reported[72] to occur in basic medium.

The aldehyde in THF was treated with 5N aqueous NaOH. The product which was presumably [CCXXII] was reduced with sodium borohydride giving an alcohol as the major product. The mass spectrum of this alcohol did not show any significant difference from the mass spectrum of [CCXX]. It was therefore not clear at this stage whether or not epimerization occurred.

Removal of the acetonide protecting group in methanol containing a catalytic amount of p-toluene sulphonic acid gave an alcohol which was presumably [CCXXIII]. The mass spectrum (Fig. 26) of the TMSi ether of [CCXXIII] was similar to the mass spectrum of the TMSi ether of CCXVII. The relative intensities of some of the ions were however different. In these respects the mass spectrum of the TMSi ether of CCXXIII resembled the mass spectrum of the MeTMSi of the natural compound (gibberellin Y) more closely than the mass spectrum of the TMSi ether of CCXVII. The differences seemed to suggest that the epimerization of the 17-CH_2OH group was achieved in CCXXIII. That is the structure of the triol [CCXXIII] was as shown.

In order to be certain that CCXXIII was actually prepared, it was decided to attempt its synthesis via another scheme (scheme 43). The method was similar in principle to the method used by Croft *et al*[190] to prepare *ent*-7α, 17-dihydroxykauran-19-oic acid [CCXXIV].

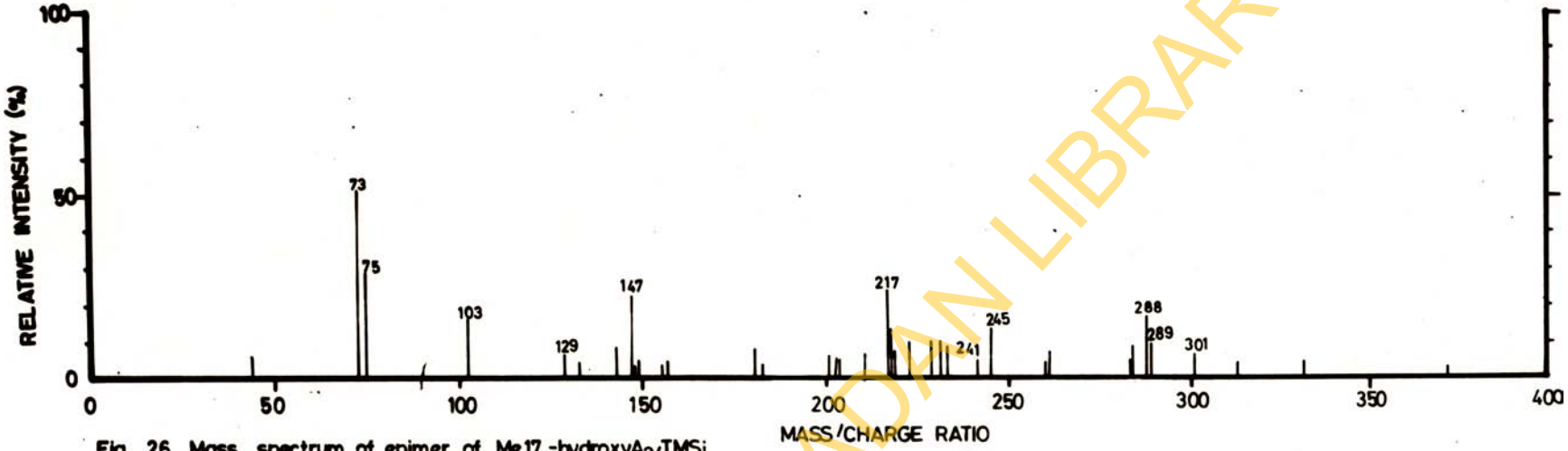


Fig. 26 Mass spectrum of epimer of Me17 -hydroxyA_{34}TMSi

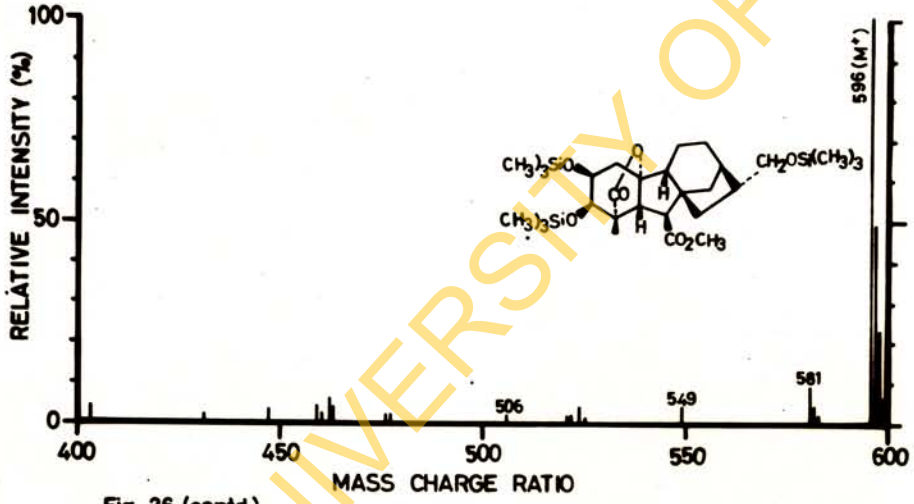
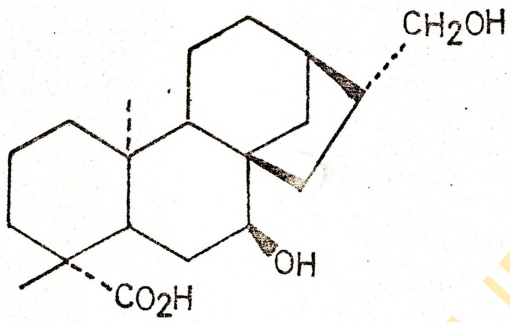
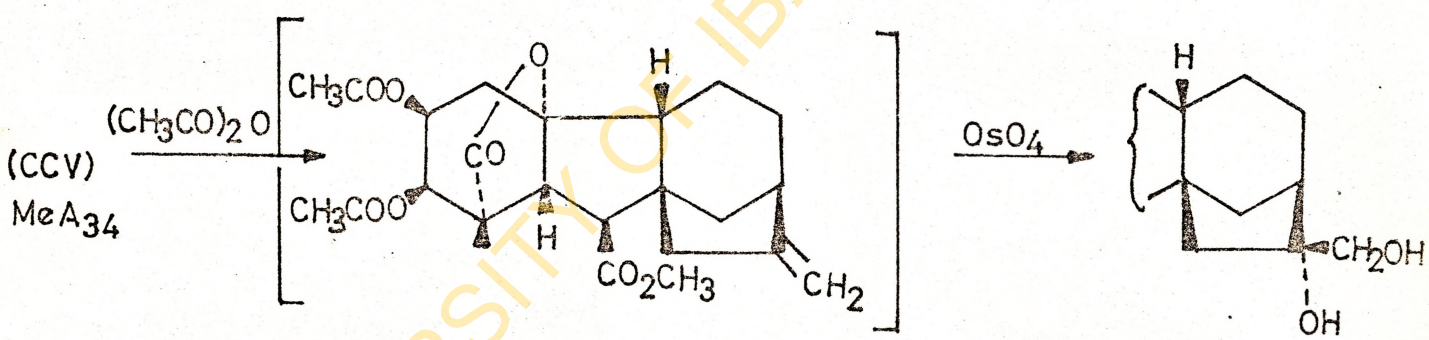


Fig. 26 (contd.)



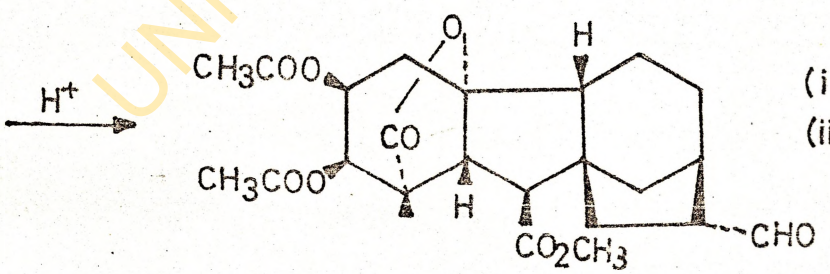
(CCXXIV)

SCHEME 43



(CCXXV)

(CCXXVI)



(CCXXVII)

(i) $NaBH_4$
(ii) OH^-

(CCXXIII)

The 2,3-diol system of MeA_{34} [CCV] was protected as the diacetate by dissolving the MeA_{34} in acetic anhydride containing a catalytic amount of p-toluene sulphonic acid at room temperature.

Acetylation is a widely used method for protecting hydroxyl groups against reactions proceeding under acidic conditions[191,192].

The product of the acetylation [CCXXV] was treated without further analysis with osmium tetroxide followed by the hydrolysis of the resultant osmate ester to give the 16,17-diol [CCXXVI]. Osmylation reactions are known to proceed from the less hindered side of a molecule. It followed therefore that an attack from the less hindered α side of the ring D of [CCXXV] should result in the 16α, 17-dihydroxy compound [CCXXVI].

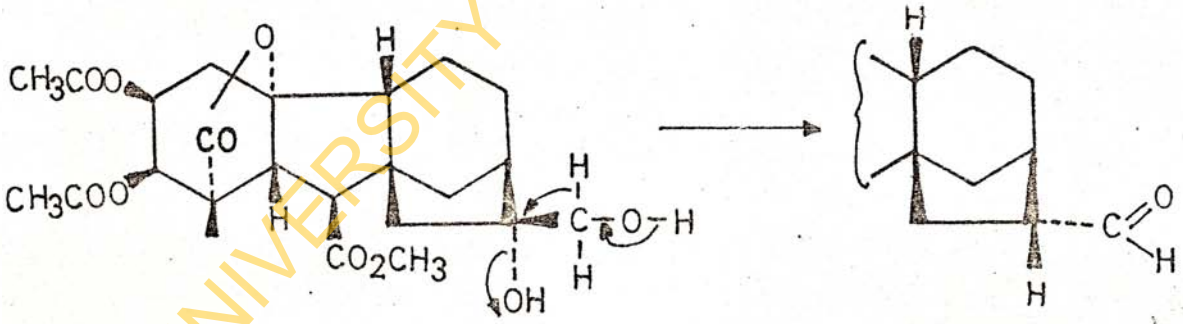
GC-MS analysis of the product (as the TMSi ether) of the osmylation reaction indicated that CCXXVI was the main product. The mass spectrum of the TMSi derivative had a molecular ion at m/e 624. Some other prominent ions were at m/e 609 (M^+-CH_3), m/e 521 (M^+-CH_2 OTMSi), m/e 403 (M^+-CH_2OTMSi, $2CH_3COO$), m/e 73 and 75 (TMSi).

The next stage which was the dehydration of the 16, 17-diol system was effected by refluxing the diol in a mixture of 1-2% conc. H_2SO_4 in THF for about 30 mins. Analysis of the product on the GC-MS indicated the presence of the aldehyde [CCXXVII].

The mass spectrum of the aldehyde had significant ions at m/e 460 (M^+-2), 431 (M^+-CH_3O), 420 (M^+-CH_2CO), 402 (M^+-CH_3COOH), 298 (M^+-$2CH_3COOH,CO_2$), 238 (M^+-$3CH_3COOH,CO_2$) and m/e 43 (base peak) (CH_3CO).

The mechanism of the formation of the aldehyde from the diol might be as shown in scheme 44 below. The acid induced elimination of the 16-hydroxyl group with the concerted rearrangement shown would lead to the formation of the aldehyde [CCXXVII].

SCHEME 44



(CCXXVI) (CCXXVII)

According to the report by Croft et al[190] the dehydration was accompanied by the epimerization of the aldehyde group in the acid medium. The epimerization was also assumed to have occurred in this case since the same conditions were employed.

Reduction of the aldehyde with sodium borohydride and the deacetylation of the 2,3-hydroxyl groups by treatment with 5% potassium methoxide gave an alcohol. The TMSi derivative of the alcohol was analysed on the GC-MS. The mass spectrum was identical to the mass spectrum of the TMSi of the alcohol [CCXXIII] obtained in scheme 42. This thus seemed to indicate that epimerisation of the 17-hydroxymethyl group was achieved through both schemes 42 and 43.

Thus it followed that the structure [CCIIb] postulated for gibberellin Y was wrong. Gibberellin Y could also not have been just the 16-epimer of [CCIIb]. However the mass spectra of the TMSi ethers of the two synthetic compounds CCXVII and CCXXIII were quite similar to the mass spectrum of the MeTMSi of the natural compound. This suggested that their structures were closely related.

On the basis of their mass spectra, the MeTMSi of gibberellin Y appeared to be more structurally related to the TMSi of CCXXIII than the TMSi of CCXVII. It was therefore thought that in the natural compound (gibberellin Y) the 17-methylene hydroxyl group might have

the α configuration as in CCXXII. The difference might therefore be in the orientation of either or both of the 2,3-hydroxyl groups.

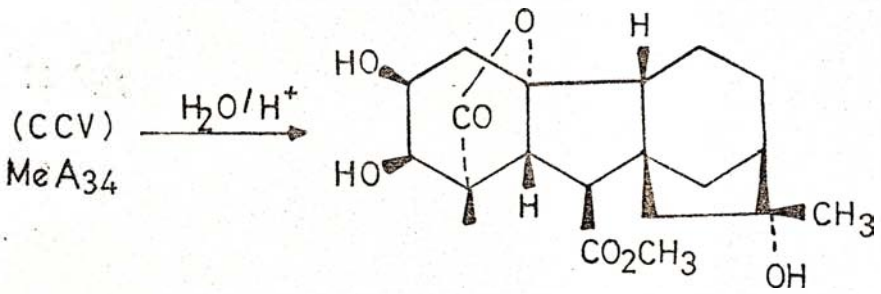
2. Partial Synthesis of 16-hydroxy MeA_{34}

It was thought that it was very unlikely that gibberellin Y was 16-hydroxy GA_{34}. This was because the mass spectrum (Fig. 19, page 149a) of the MeTMSi of gibberellin Y did not have a prominent ion at m/e 130. An intense ion at m/e 130 [XXIX] is characteristic of the mass spectra of the MeTMSi of 16-hydroxylated gibberellins. It was however decided to synthesize 16-hydroxy $MeGA_{34}$ [CCXX] in order to compare the mass spectrum of its TMSi ether with that of the MeTMSi ether of gibberellin Y.

The exocyclic 16,17-double bond of gibberellins which lack 13-hydroxylation is readily hydrated with dilute aqueous acid to form the 16-α hydroxyl derivative[31,193]. This method has been used to synthesize GA_2 [XVII] from GA_4 [XIV][31].

Treatment[31] of MeA_{34} [CCV] with aqueous 3N HCl at room temperature gave the 16-α hydroxy MeA_{34} [CCXXVIII] as the major product.

SCHEME 45



(CCXXVIII)

The stereochemistry of the 16-hydroxyl group was assumed to be α because the reagent was expected to attack the double bond from the less hindered α face of ring D. Similar hydration of some other gibberellins that have been reported[31,193] gave the 16α-hydroxyl derivatives of these gibberellins.

The mass spectrum of the TMSi ether of the 16α-hydroxyMeA$_{34}$ had a molecular ion at m/e 596. There was the prominent ion (base peak) at m/e 130 indicating the presence of the 13-hydroxyl group. There were prominent ions at m/e 147 and 217 indicative of the 2,3-diol system. The mass spectrum was quite distinct from the mass spectrum of the MeTMSi of gibberellin Y. Therefore the natural compound could not have been 16α-hydroxyGA$_{34}$.

CONCLUSIONS

The results of the comparative analysis of some of the hormones in the acidic ethyl acetate extracts obtained from 6-day old fruits (from the lowest raceme) and 2-day old fruits (from the upper racemes) showed that there was a disparity in the hormonal contents of these two groups of fruits. The 6-day old fruits had both growth promoting hormones (gibberellins and auxins) and growth inhibiting hormones (abscisic acid and its metabolites). Only the growth inhibiting hormones could however be identified in the 2-day old fruits. It can therefore be inferred that the abscission of the younger, upper fruits of the cowpea inflorescence is due to the lack of adequate concentration of growth promoting hormones in these fruits. Since they have no detectable amount of growth promoting hormones to counteract the effect of the growth inhibitors, their growth will be arrested and they will abscise. Also, the older fruits at the lowest raceme will monopolise the available nutrients since they have an adequate concentration of growth promoting hormones which will induce the mobilization[8] of the available nutrients towards these fruits.

The inducing influence which the maturing fruits at the first (lowest) raceme have on the abscission of the younger, upper fruits can therefore be partly due to the monopolization of the

available nutrients by the oldest (lowest) fruits. This agrees with the observations of Adedipe and Ormrod[6] and Adedipe et al[7] that competition for nutrients is an important factor in the abscission problem in cowpea.

The inducing influence which the oldest (lowest), fruits have on the abscission of the younger, upper fruits can also be partly due to the abscising influence of hormones from the oldest, basal fruits which are translocated along the peduncle in the acropetal (apical) direction. This agrees with Ojehomon[2]'s hypothesis that the abscission of the flower buds and immature fruits may be stimulated by a substance or substances produced in the older fruits.

The results of the GC-MS analysis of the crude acidic ethyl acetate fractions obtained from 6-day old seeds and 6-day old fruit walls clearly showed that most of the impurities that interfered with the analysis were from the fruit walls and not from the seeds. It therefore appears advisable that any further investigations of the hormonal content of cowpea fruits ought to be concentrated on the seeds despite the difficulties encountered in separating the tiny seeds from the fruit walls.

EXPERIMENTAL

General

Melting points were determined on a Koefler hot-stage apparatus and were not corrected. Solvents were redistilled prior to use. Petroleum ether refers to light petroleum b.p. 60-80°.

The following chromatographic materials were used for column chromatography: activated charcoal (BDH), Celite 545, (Johns-Manderville) and silica gel MFC (Hopkins and Williams). For preparative thin layer chromatography, plates were prepared with Kiesel gel G, HF or PF (Merck) and were pre-eluted with ethyl acetate. For analytical thin layer chromatography, plates (0.25mm) were prepared with kiesel gel G (Merck). After development they were viewed in ultra-violet light after spraying with five per cent sulphuric acid in ethanol and heating in the oven.

Nuclear magnetic resonance (n.m.r) spectra were obtained with a Varian HA 100 spectrometer for deuterochloroform or deuteropyridine solutions with tetramethylsilane as internal standard. N.m.r. absorptions are quoted in δ units.

Methylation was carried out by addition of excess ethereal diazomethane to solutions of the compounds or extracts to be methylated in a minimum amount of dry methanol. Trimethylsilyl (TMSi) derivatives of the methyl esters were prepared by adding a

silylating reagent[194] consisting of pyridine, hexamethyldisilizane and trimethylchlorosilane (2:2:1, v/v/v) to solutions of the previously dried methylated extracts or compounds in dry pyridine. In each case the resulting mixture was usually left standing in a sealed tube at room temperature for about thirty minutes before analysis on the combined gas chromatograph — mass spectrometer (GCMS). In some cases the mixture in the sealed tube was warmed slightly with a hair-dryer for a few seconds.

Gas chromatography and combined gas chromatography - mass spectrometry

The methyl esters (Me) and methyl ester trimethyl silyl ethers (MeTMSi) were chromatographed on a Pye 104 gas chromatograph using 2% SE 33 on Gas chrom Q (80 - 100 mesh) packed in a glass column (130cm x 0.4cm i.d.). Column temperatures were usually programmed from 180° to 250° at 4° per minute with a nitrogen flow rate of 60ml per minute.

Gas chromatography - mass spectrometry (GCMS) analysis of the methyl esters and the MeTMSi derivatives was done on an A.E.I.-G.EC. MS 30 mass spectrometer coupled to a Pye 104 gas chromatograph through a silicone membrane. The glass column (171cm x 0.2cm i.d.) for the GCMS was packed with 2% SE 33 (or sometimes 2% QFI) on Gas Chrom Q (80-100 mesh) with a helium flow rate of 25ml per minute.

The mass spectra were obtained at 24 ev and were processed on-line by a computer.

Materials and extraction procedures

The 'New Era' cultivar of cowpea was used. The seeds that were sown and harvested for extraction were obtained from the National Cereals Research Institute, Moor Plantation, Ibadan.

The seeds were grown in the field at spacings 91 x 30cm (3 x 1 ft.) and this enabled the plants to grow as individuals. Flowers were labelled on the day they opened in order to determine the age of each fruit. The fruits were frozen in liquid nitrogen and then (unless otherwise stated) freeze dried.

(a) Extraction of 2-day old fruits

The fruits (40g. dry weight or 200g. fresh weight) were ground in a Waring blender in 80 per cent aqueous methanol ($15cm^3$/g. dry weight of plant material). The mixture was left overnight in the cold room (ca.$0^{\circ}C$) and was then filtered. The residue was mixed with fresh 80% aqueous methanol and left in the cold room for twenty-four hours before filtration. The extraction of the residue with 80% aqueous methanol was repeated twice (24 hours each time). The combined filtrates (2.2 lites) were concentrated (ca.$300cm^3$) at below $40^{\circ}C$ under reduced pressure in order to remove the methanol.

The pH (5.2) of the concentrated aqueous extract was adjusted to between 8.0 and 8.1 with 2M sodium hydroxide solution. The aqueous extract was then extracted with petroleum ether (3 x 100cm^3). The petroleum ether layer was discarded.

The aqueous layer was extracted with ethyl acetate (3 x 100cm^3). The ethyl acetate layer was backwashed with water (100cm^3) and the water was added to the aqueous layer. The ethyl acetate layer was evaporated at below 40^{o}C under reduced pressure giving the neutral ethyl acetate (NE) fraction as a gum (213mg).

The pH of the aqueous layer was adjusted to 3.0 with 2M aqueous hydrochloric acid. The aqueous layer was then extracted with ethyl acetate (4 x 150 cm^3). The ethyl acetate layer was backwashed with water (150cm^3) and the water was added to the aqueous layer. The ethyl acetate layer was evaporated at below 40^{o}C under reduced pressure to give the acidic ethyl acetate (AE) fraction as a gum (430mg).

The aqueous layer was extracted with water-saturated ('wet') n-butanol (3 x 150cm^3). The n-butanol layer was backwashed with water (150 cm^3) and the water was added to the aqueous layer. The n-butanol layer was evaporated at below 40^{o}C under reduced pressure to give the acidic n-butanol (AB) fraction as a brown gum (838mg).

The aqueous layer was discarded.

The backwash of the various extracts with water was to remove residual acid or base.

(b) Extraction of 6-day old fruits

The fruits (110g. dry weight or 660g fresh weight) were ground in a blender in 80 per cent aqueous methanol ($15cm^3$/g dry weight of plant material). The mixture was left overnight in the cold room, filtered and the residue extracted thrice more with 80 per cent aqueous methanol. The combined filtrates (ca. 6.5 litres) were concentrated (ca.$600cm^3$) at below 40^oC under reduced pressure.

The pH (4.8) of the concentrated aqueous extract was adjusted to between 8.0 and 8.1 with 2M sodium hydroxide and the extract was washed with petroleum ether (3 x $200cm^3$). The petroleum ether extract was discarded.

The aqueous layer was then extracted with ethyl acetate (3 x $200cm^3$). After backwashing with water ($200cm^3$), evaporation of the ethyl acetate layer under reduced pressure at below 40^oC gave the neutral ethyl acetate (NE) fraction as a gum (526mg).

The pH of the aqueous layer was adjusted to 3.0 and the aqueous layer was then extracted with ethyl acetate (4 x $250cm^3$). The ethyl acetate layer was evaporated in the usual way (after backwashing with water) to give the acidic ethyl acetate (AE) fraction as a gum (700mg.)

The aqueous layer was extracted with 'wet' n-butanol (3 x 250cm^3). After hackwashing with water (200cm^3), the n-butanol layer was evaporated to give the acidic n-butanol (AB) fraction as brown gum (1.4g).

(c) Extraction of 6-day old seeds

Seeds were removed from 6-day old fruits. The seeds (21g. fresh weight) were ground in a blender in 80 per cent aqueous methanol. The extraction procedure was then carried out as described above for 6-day old fruits, giving an acidic ethyl acetate fraction (18mg) as a brown gum.

(d) Extraction of 6-day old fruit walls

The 6-day old fruit walls (34g fresh weight) were extracted by the same procedure described above for the extraction of 6-day old fruits giving an acidic ethyl acetate fraction (80mg) as a brown gum.

(3) Another extraction[63] of 6-day old fruits (used for paper chromatography)

The 6-day old fruits (5 kg. fresh weight) were ground in a mill and extracted thrice with 70 per cent aqueous methylated spirit. The combined extract (ca.5.5 litres) was concentrated (ca.700cm^3) under reduced pressure at below 40°C.

The pH (5.0) of the concentrated aqueous extract was adjusted to between 7.3 and 7.4 with 1M sodium carbonate solution. The aqueous extract was then extracted with ethyl acetate (3 x 250cm^3). Evaporation of the ethyl acetate layer *in vacuo* gave the neutral ethyl acetate fraction.

The pH of the aqueous layer was adjusted to 3.0 with 3M hydrochloric acid, and the aqueous layer was extracted with ethyl acetate (4 x 250cm^3). The ethyl acetate layer was backwashed with water until the washings were neutral. The ethyl acetate layer was then concentrated (ca.300cm^3) and extracted with phosphate buffer[195] pH 6.3 (4 x 100cm^3). Evaporation of the ethyl acetate layer gave the weak acidic ethyl acetate fraction.

The pH of the phosphate buffer layer was adjusted to 3.0 with 3M hydrochloric acid the phosphate buffer layer was then extracted with ethyl acetate (4 x 150cm^3). After backwashing with water, the ethyl acetate layer was evaporated *in vacuo* giving the strong acidic ethyl acetate fraction as a gum (1g).

Another modification of the procedure used for the extraction of 6-day old fruits was carried out as described below.

Freeze-dried 6-day old fruits (126g dry weight) were ground and then extracted in a soxhlet extractor with 80% aqueous methanol. The flask containing the aqueous methanol was immersed in a water bath

maintained at 35°C. In order to bring the aqueous methanol to reflux, the condenser on top of the soxhlet extractor was connected to a vacuum pump. The condenser was cooled with cold methylated spirit (ca. - 15°C) circulated from a cooler. The extraction was carried on until no more chlorophyll was extracted.

The aqueous methanol extract (1.5 litres) was concentrated ($200cm^3$) at below 40°C under reduced pressure in order to remove the methanol.

The pH of the concentrated aqueous extract was adjusted to 7.5 with 1M sodium carbonate solution. The aqueous extract was then extracted with petroleum ether using a liquid-liquid extractor. The petroleum ether layer was discarded. The extraction procedure was then continued as described above for the extraction of 6-day old fruits (used for paper chromatography) giving a strong acidic ethyl acetate fraction as a gum (400mg). This fraction was used for some preliminary biological assays.

(f) Extraction[63] of fruits that were over 6 days old

The fruits (23kg fresh weight) were frozen in liquid nitrogen, ground in a mill and extracted in the cold with 85 per cent aqueous methanol (3 x 10 litres). The combined extracts were concentrated (ca. 3 litres) under reduced pressure.

The pH (5.6) of the aqueous extract was adjusted to 7.5 with 1M aqueous sodium carbonate solution and the aqueous extract was extracted with hexane. The pH of the aqueous extract was then adjusted further to between 8.0 to 8.1 and the aqueous layer was extracted thrice with ethyl acetate. Evaporation of this ethyl acetate layer in vacuo gave the neutral ethyl acetate fraction.

The pH of the aqueous layer was adjusted to 3.0 with 5M hydrochloric acid. The aqueous layer was then extracted with ethyl acetate (6 x 500cm^3). The ethyl acetate layer was backwashed with water (3 x 300cm^3), and dried overnight over anhydrous sodium sulphate. The dried ethyl acetate layer was then evaporated in vacuo at below 40^{o}C to give the acidic ethyl acetate (AE) fraction as a gummy solid (8g).

The aqueous layer was discarded.

Preparation of phosphate buffers.

The phosphate buffers were prepared as described below.

(a) Phosphate buffer pH 6.3

This was prepared[195] by mixing $^{M}/15$ Na_2HPO_4 solution and $^{M}/15$ KH_2PO_4 solution in a ratio of 1:4 (v/v) respectively.

The $^{M}/15$ Na_2HPO_4 solution was prepared by dissolving 9.464g of Na_2HPO_4 in 1000cm^3 of distilled water.

The M/15 KH_2PO_4 solution was prepared by dissolving 9.075g of KH_2PO_4 in 1000cm^3 of distilled water.

(b) Phosphate buffer pH 8.0

This was prepared[196] as follows:

50cm^3 of 0.2M KH_2PO_4 solution was mixed with 46.8ml 0.2M NaOH solution and the mixture was diluted to 200cm^3 with distilled water.

Purification of extracts with PVP[162]

Aliquots of the plant extracts to be purified were taken into phosphate buffer (0.2M, pH 8.0) using about 1cm^3 of buffer per extract from 2g dry weight (or 10g fresh weight) of plant material. Pre-washed polyvinylpyrrolidone (PVP or Polyclar AT) was added at concentrations ranging from 50mg to 100mg per cm^3 of phosphate buffer. Each mixture was then thoroughly shaken for thirty minutes. The PVP was filtered off under vacuum filtration and the residue washed thoroughly with aliquots of the phosphate buffer (6 x 2cm^3).

Fresh PVP was added to each filtrate. The shaking and filtration procedures were repeated two more times.

The combined phosphate buffer filtrates were washed with petroleum ether (3 x $\frac{1}{3}$ volume of buffer). The petroleum ether layer was discarded.

The buffer layer was washed with ethyl acetate (3 x $\frac{1}{3}$ volume of buffer).

The pH of the buffer layer was adjusted to pH 3.0 with 2M hydrochloric acid. The buffer layer was extracted with ethyl acetate (4 x $\frac{1}{3}$ volume of buffer). The ethyl acetate layer was backwashed with water and evaporated in vavuo to give the purified extract.

Column chromatography [63]

(a) Column chromatography of the acidic ethyl acetate fraction obtained from 2-day old fruits

Half (215mg) of the acidic ethyl acetate fraction that was obtained from 2-day old fruits was put on a column (15 x 2.5cm) of activated charcoal (16g) and celite (32g).

The column was eluted with a gradient of increasing concentration of acetone in water, obtained by connecting an aspirator of water (1.5 litres) to that of acetone (2 litres). Seventeen 150cm^3 fractions were collected. The solvent composition of the eluant for each fraction was determined by refractometry.

Selected column fractions (4 to 10) were purified with PVP, and then analysed on the GLC and the GC-MS as the methyl esters (Me) and the methyl ester trimethyl silyl ethers (MeTMSi).

(b) Column chromatography of the acidic ethyl acetate fraction obtained from 6-day old fruits

Half (350mg) of the acidic ethyl acetate fraction that was

obtained from 6-day old fruits was placed on a column (26 x 1.5cm) of activated charcoal (8g) and celite (16g).

The column was subjected to a gradient elution of increasing concentration of acetone in water. Twenty 150cm^3 fractions were collected.

Selected column fractions (4 to 10) were purified with PVP, derivatized (Me, MeTMSi) and analysed on the GLC and the GCMS.

(c) Column chromatography of the acidic ethyl acetate fraction obtained from fruits that were over 6 days old

The acidic ethyl acetate fraction (7.5g) was chromatographed on a column (54 x 4.5cm) of activated charcoal (70g) and celite (140g).

The column was eluted with an increasing gradient of acetone in water, obtained by connecting an aspirator of water (4 litres) to another aspirator containing acetone (5 litres).

Forty fractions were collected. The first two were 500cm^3 fractions while the others were 200cm^3 fraction.

Some fractions, which were selected on the basis of the results that were obtained for the analytical TLC and the 'halo' bioassay carried out on the column fractions, were derivatized (Me, MeTMSi) after purification with PVP. The derivatized fractions were then analysed on the GLC and the GC-MS.

Thin-layer chromatography (TLC) of the acidic ethyl acetate (AE) fractions for wheat coleophtile segment bioassay

Aliquots of the PVP purified AE fractions from 2-day and 6-day old fruits (equivalent to extract from 20g. fresh weight of fruits) were each applied on a TLC plate (20 x 20cm) which was prepared with kiesel gel G and had been pre-developed in ethyl acetate and dried. Each plate was then developed in ethyl acetate: chloroform: acetic acid (15: 5: 1, v/v/v) to a distance of 15cm. The zone of development was divided into ten equal Rf zones. Silica gel from each Rf zone was eluted with 'wet' ethyl acetate. The silica gel from a zone below the base-line was also eluted with 'wet' ethyl acetate and the extract from this zone was used as the control.

Half of the extract from each zone was tested for biological activity in the wheat coleoptile segment bioassay described below.

Paper chromatography of the AE fraction (from 6-day old fruits) for the wheat coleoptile segment bioassay

After purification with PVP, aliquot of the acidic ethyl acetate fraction (equivalent to extract from 5g. fresh weight of fruits) obtained from 6-day old fruits was strip-loaded on a 10mm wide strip of Whatman No. 1 filter paper. The paper had been pre-run first in ethanol and then in a solvent mixture consisting of isopropanol: ammonia (25%): water (10: 1:1, v/v/v). After the

extract had been strip-loaded on the paper, the paper was equilibrated overnight with the solvent mixture (isopropanol: ammonia: water) before it was developed in the same solvent mixture. The development was descending to a distance of about 18cm.

After drying, the zone of development was cut into ten equal segments and two segments were also cut in the area below the base-line. The last two segments were used as controls. The various Rf zones and the controls were then tested for biological activity in the wheat coleoptile segment bioassay as described below.

Bioassays

(a) Wheat coleoptile segment bioassay[95]

Wheat grains (cultivar Kolibri) were kept in running water for about 24 hours and then planted in vermiculite in a dark chamber at room temperature. After about three days, coleoptiles which were between 18 mm to 23 mm long were selected. A 10mm segment was cut at 2mm from the tip of each coleoptile. The selections and cuttings were carried out in the dark room with a dim yellow light. The 10mm coleoptile segments were left floating in distilled water in the dark for about two hours. The soaking in water was to reduce the concentration of the endogenous hormones of the coleoptile segments.

For each assay, five coleoptile segments were incubated with each paper chromatogram Rf zone or with the aliquot of the extract from each TLC Rf zone in a specimen bottle containing $1.5cm^3$ of a buffer solution.

The buffer solution that was used as the medium was prepared as follows: A stock solution containing 0.1M K_2HPO_4 and 0.05M citric acid monohydrate was prepared by dissolving 4.485g of K_2HPO_4 and 2.547g of citric acid monohydrate in $250cm^3$ of distilled water. 2g of sucrose was added to $10cm^3$ of the stock solution and the mixture was diluted to $100cm^3$ with distilled water. $1.5cm^3$ of this solution was used as the medium in each specimen bottle.

A tiny hole was punched in the centre of the plastic lid of each specimen bottle to ensure adequate supply of air to the coleoptile segments.

The specimen bottles with their contents were rotated horizontally about their main axis in the dark for about 24 hours. The rotation was to prevent coleoptile segments curvatures caused by gravitational stimulation. The lengths of the coleoptile segments were measured after 22 hours.

(b) Halo bioassay[163]

Barley (Hordeum vulgarum, cultivar, White naked atlas) seeds were cut transversely into two. The embryo sections were discarded.

The embryoless (endosperm) half-seeds were soaked for twenty minutes in 1% (v/v) sodium hypochlorite in water. After rinsing thoroughly in sterile water, the half-seeds were left to imbibe water in a beaker for about 20 hours.

Meanwhile a mixture consisting of 3% agar and 0.25% soluble starch in water (w/v) was prepared. The mixture was autoclaved for about twenty minutes.

Aliquots (1 cm^3 aqueous ethanol solutions- equivalent to extract from 5g fresh weight of fruits) of the column fractions to be tested were put in 9cm petri dishes. 25cm^3 of the autoclaved agar mixture were added to each petri dish and the mixture was shaken thoroughly. The mixture was then left to set.

The barley half-seeds were then placed on the agar mixture (with the cut surface on the mixture) in the petri dishes. The petri dishes and their contents were left at about 20°C for two days.

After the two-day incubation period, a potassium iodide-iodine solution (prepared by dissolving 2.0g of potassium iodide and 200mg of iodine in 100cm^3 of water) was added to the mixture in each petri dish.

The presence of active gibberellins in the materials tested was indicated by the presence of a clear circle (halo) around each half-seed. The diameter of each clear circle was measured as a quantitative estimation of the active gibberellins present.

For each test controls were prepared consisting of 1 cm^3 aqueous ethanol and 25cm^3 agar-starch mixture. Standard gibberellin A_3 consisting of 2$\mu g/cm^3$ was also tested.

Synthesis

ent-10-Hydroxy-20-norgibberell-2,16-diene-7,19-dioic Acid 7-Methyl ester 19,10-Lactone (2,3-dehydroMeGA_9, CCIV)

A GA_4/GA_7 mixture (7:3; 300mg) was dissolved in a little methanol and methylated with ethereal diazomethane.

The methylated mixture without further purification was dissolved in pyridine (7cm^3) and treated with phosphorus oxychloride (0.7cm^3). The reaction mixture was left at room temperature for 12 hours and then refluxed for 3 hours. On cooling, the reaction mixture was added to water slowly. The mixture was acidified (pH 3.5) with concentrated hydrochloric acid and it was then extracted with ethyl acetate. The ethyl acetate extract was backwashed with water and evaporated under reduced pressure giving a gummy product (245mg).

The gummy product was purified on preparative TLC (silica gel G, 0.9mm). After development (twice) in ethyl acetate-petroleum ether (20 : 80, v/v), silica gel from Rf zone 0.5 to 0.55 was eluted with ethyl acetate giving the olefin, CCIV, as colourless

crystals (85mg), m.p. 125-130° (recrystallised from ethyl acetate-petroleum ether). δ ($CDCl_3$ + TMS) 1.23 (3H, S, 18-H_3), 2.65 (1H, d, 5-H), 2.83 (1H, d, 6-H), 3.73 (3H, S, CO_2CH_3), 4.88 and 4.99 (each broad, 17-H_2) and 5.75 (2H, m, 2-H and 3-H). m/e 328 (M^+, < 1%), 284 (40), 225 (14), 224 (100), 223 (21), 216 (16), 209 (28), 181 (32), 157 (26), 156 (42), 155 (28), 151 (30), 143 (23), 131 (21), 119 (25), 105 (62), 93 (42), 92 (31), 91 (39), 79 (28), and 44 (24).

ent - 2α, 3α, 10, 16β, 17-Pentahydroxy-20-norgibberellane-7,19-dioic Acid 7-Methyl ester 19,10-Lactone (16α, 17-dihydroxyMeGA$_{34}$, CCIII)

2,3-dehydrogibberellin A_9 methyl ester, CCIV (50mg) was dissolved in pyridine (2cm^3) and the stirred solution was treated[174] with osmium tetroxide (100mg). The stirring was continued at room temperature for 2 days (when analytical TLC showed no starting material).

The reaction mixture was stirred and treated[175] slowly with a solution of sodium metabisulphite (400mg) in water (6 cm^3) and pyridine (4.5 cm^3). The mixture was stirred for a further thirty minutes. The mixture was neutralised with concentrated hydrochloric acid and extracted with ethyl acetate. The ethyl acetate extract was backwashed with water and then evaporated in vacuo to give a solid (40mg).

The solid was purified on preparative TLC (silica gel G, 0.4mm), developed twice in ethyl acetate-chloroform-acetic acid (15: 5: 1, v/v/v). Silica gel from Rf zone 0.15 to 0.25 was eluted with ethyl acetate-methanol (9:1, v/v) giving the tetrol, CCIII as a solid (28mg). It was recrystallized from ethyl acetate, m.p. 130-140°. δ (C_5D_5N + TMS) 1.49 (3H, S, 18-H_3), 3.05 (1H, d, 6-H), 3.60 (3H, S, CO_2CH_3), 3.78 (1H, d, 5-H), 3.97 (2H, d, 17-H_2), 4.13 (1H, d, 3-H), 4.32 (1H, m, 2-H). The TMSi derivative was prepared and the mass spectrum (Fig. 24) was run. m/e 684 (M^+, 7%), 583 (23), 582 (48), 581 (100), 218 (4), 217 (9), 147 (14), 143 (4), 129 (9), 103 (3), 75 (22) and 73 (30).

ent-3α, 10-Dihydroxy-16-0xo-17,20-bisnorgibberellane-7,19-dioic Acid 19,10 Lactone (GA_4 norketone, CCVI)

A stirred ice-cold solution of GA_4/GA_7 mixture (7:3, 3g) and osmium tetroxide (20mg) in tetrahydrofuran (THF, 45cm^3) and water 45cm^3 was prepared. Powdered sodium metaperiodate (3.75g) was added slowly, (20 minutes) in small portions, to the stirred solution. The mixture was allowed to warm to room temperature and the stirring was continued for 24 hours. The mixture was concentrated in vacuo to remove the THF. It was then diluted with more water and acidified (pH 3.0) with dilute hydrochloric acid. It was extracted with ethyl acetate and the ethyl acetate layer was backwashed with

water to remove the residual hydrochloric acid. Evaporation of the ethyl acetate layer gave the crude gibberellin A_4 norketone (CCVI, 2.0g). This was recrystallised from ethyl acetate-petroleum ether m.p. 120-136° (Lit.[179] m.p. 120-140°).

ent-10-Hydroxy-16-Oxo-17,20-bisnorgibberell-2-ene-7,19-dioic Acid 7-Methyl ester 19,10-Lactone (2,3-dehydroGA_9 norketone methyl ester CCVII)

The gibberellin A_4 norketone, CCVI (1.8g) was dissolved in methanol (5 cm^3) and methylated with excess ethereal diazomethane. Evaporation of the mixture gave the methyl ester of gibberellin A_4 norketone.

The gibberellin A_4 norketone methyl ester, without further purification was dissolved in pyridine (45 cm^3) and treated with phosphorus oxychloride (4.5cm^3). The mixture was left standing at room temperature overnight and was then refluxed for about 3 hours.

The reaction mixture, on cooling, was added slowly to water with stirring and then acidified with concentrated hydrochloric acid to pH 4.0. The mixture was extracted with ethyl acetate and the ethyl acetate layer was backwashed with water. Evaporation of the ethyl acetate layer *in vacuo* gave a solid (1.7g).

The solid was purified by column chromatography on silica gel. Elution with 30% ethyl acetate in petroleum ether gave the 2,3-dehydroGA_9 norketone methyl ester (CCVII). This was recrystallised

in 50% ethyl acetate: petroleum ether (v/v) to give colourless crystals (700mg) m.p. 158-160° (Literature[43,73] m.p. 158-159°, 160-161°). δ ($CDCl_3$ + TMS) 1.23 (3H, S, 18-H_3), 2.5 (1H, d, 5-H), 2.68 (1H, d, 6-H), 3.73 (3H, d, CO_2CH_3), 5.6 to 5.9 (2H, m, 2-H and 3-H). This was consistent with the n.m.r spectrum reported in literature[176] for the compound.

m/e 330 (M^+, < 1%), 286 (34), 227 (44), 226 (100), 183 (35), 105 (32), and 95 (33).

ent-2α, 3α,10-Trihydroxy-16-Oxo-17,20-bisnorgibberellane-7,19-dioic Acid 7-Methyl ester 19,10-Lactone (MeA_{34} norketone, CCVIII)

The 2,3-dehydroGA_9 norketone methyl ester, CCVII (500mg) was dissolved in pyridine (20cm^3). The stirred solution was treated with osmium tetroxide (500mg). The stirring was continued at room temperature for two days.

The reaction mixture was stirred and treated slowly with a solution of sodium metabisulphite (4g), water (60cm^3) and pyridine (45cm^3). The mixture was stirred for a further thirty minutes after the addition.

The mixture was neutralised with concentrated hydrochloric acid and extracted with ethyl acetate. The ethyl acetate layer was backwashed with water and then evaporated to give a white solid (482mg).

The solid was recrystallised from methanol to give the MeA_{34} norketone, CCVIII (350mg). M.p. 113-117° (Lit.[43] m.p. ca. 115-120°).

It was then converted to the TMSi ether and its mass spectrum run. m/e 509 (40%), 508 (M^+, 100), 218 (26), 217 (45), 147 (36), 75 (23) and 73 (49).

ent-2α, 3α,10-Trihydroxy-20-norgibberell-16-ene-7,19-dioic Acid 7-Methyl ester 19,10-Lactone (MeA_{34}, CCV)

The trimethylsilyl ether of $MeGA_{34}$ norketone, CCIX was prepared by adding a silylating reagent (500µl) to a solution of the methyl ester of GA_{34} norketone (100mg) in dry pyridine (100µl) in a tightly covered screw-cap pressure vial. The silylating reagent was made up of hexamethyldisilazane, trimethylchlorosilane and pyridine (2: 1: 2, v/v/v). The pressure vial and its contents were warmed slightly with a hair dryer for a few seconds and then left standing at room temperature for about 1 hour. The mixture was evaporated and extracted with dry ethyl acetate. The ethyl acetate extract was centrifuged and the supernantant was evaporated to give the trimethylsilyl ether of the methyl ester of gibberellin A_{34} norketone, CCIX as a solid. This was dried thoroughly under vacuum. m/e 509 (40%), 508 (M^+, 100), 243 (11), 225 (11), 218 (26), 217 (45), 147 (36), 75 (23) and 73 (49).

Triphenylphosphine (11g) was dissolved in dry benzene (9 cm^3) and the resulting solution was cooled in an ice-salt mixture. To the cooled solution in a screw-cap bottle was added pre-cooled methyl bromide (3.23cm^3 ≡ 5.6g). The bottle was covered tightly and left to stand at room temperature for 24 hours. The resulting white solid was collected by suction and washed with hot benzene (ca.200cm^3) to give methyltriphenyl phosphonium bromide (14.9g). This was dried at 100° for 24 hours. M.p. 233 - 234°. (Lit.[197] m.p. 232 - 233°).

Methyltriphenyl phosphonium bromide (3g) was suspended in 30cm^3 of dry peroxide-free tetrahydrofuran. Sodium hydride (400mg) was added to the mixture with stirring under a flow of nitrogen. The mixture was heated until a yellowish green colour was obtained. Stirring was continued for 24 hours under a flow of nitrogen at room temperature. After 24 hours, the stirring was stopped and the sodium bromide was allowed to settle. Aliquot (4cm^3) of the supernantant (methylenetriphenyl phosphorane in THF) was added to a solution of the trimethylsilyl ether of the methyl ester of GA_{34} norketone (prepared from 100mg of $MeGA_{34}$ norketone as described above) in a small amount of dry tetrahydrofuran. The addition was carried out with stirring under nitrogen and the stirring was continued for 24 hours after the addition.

The product was evaporated under nitrogen and the resulting crude trimethylsilyl ether of the methyl ester of GA_{34} was hydrolysed with acetic acid-methanol (4:1), ($5cm^3$) at room temperature for about 12 hours (TLC monitoring). The resulting mixture was evaporated *in vacuo* after the addition of water and toluene (to remove the acetic acid azeotropically). The residual gum was purified on preparative TLC on kiesel gel with ethyl acetate-petroleum ether (15: 5, v/v) giving gibberellin A_{34} methyl ester, CCV, as a gum (25mg). $\delta(CDCl_3 + TMS)$ 1.18 (3H, S, 18-H_3), 3.02 (1H, d, 6-H), 3.25 (1H, d, 5-H), 3.70 (3H, S, CO_2CH_3), 4.86 and 4.96 (17-H_2).

The TMSi derivative was prepared and the mass spectrum run. m/e 507 (43%), 506 (M^+, 100), 241 (11), 233 (10), 229 (14), 223 (17), 218 (15), 217 (30), 210 (10), 147 (31), 75 (35) and 73 (58).

Woodward reaction[177] on gibberellin A_{34} methyl ester

Silver acetate (4.67mg, 0.28mM) was added to a stirred solution of gibberellin A_{34} methyl ester, CCV (5mg, 0.14mM) in acetic acid ($1cm^3$). Finely powdered iodine (3.55mg, 0.14mM) was added in small portions to the vigorously stirred mixtured for about thirty minutes. Aqueous glacial acetic acid (100µl) was then added. (The aqueous glacial acetic acid was prepared by diluting 20µl of water to 500µl with glacial acetic acid.) The reaction mixture was heated at

90-95°C with stirring for 3 hours. The mixture was allowed to cool, diluted with more water, and extracted with ethyl acetate. The ethyl acetate layer was backwashed with a little water and evaporated to give a gum (5.4mg).

The gummy product (4mg) obtained above was hydrolysed by treating a solution of the gum in a little methanol with 5% potassium hydroxide in methanol overnight. The mixture was neutralised with dilute hydrochloric acid, more water was added, and the mixture was concentrated. The concentrated mixture was extracted with ethyl acetate and the ethyl acetate layer was evaporated (after backwashing with water) to give a gum (2.5mg) GC-MS analysis of the TMSi derivative of the gum revealed that it was a mixture of several compounds. The mass spectra of three of the GLC peaks had the expected molecular ion at m/e 684.

m/e 684 (M^+, 9%), 583 (21), 582 (45), 581 (100), 491 (7), 490 (13), 217 (8), 147 (18), 143 (6), 129 (5), 75 (10), and 73 (26).

m/e 684 (M^+, 7%), 596 (11), 595 (16), 594 (36), 583 (20), 582 (40), 581 (100), 540 (13), 491 (13), 490 (36), 218 (12), 217 (28), 156 (27), 147 (39), 143 (13), 129 (9), 103 (4), 75 (27) and 73 (72).

m/e 684 (M^+, 7%), 634 (13), 633 (27), 632 (65), 594 (33), 582 (34), 581 (66), 540 (28), 522 (19), 491 (19), 490 (27), 456(13), 287 (13), 218 (25), 217 (57), 156 (33), 147 (61), 143(21), 129(19), 103(9), 75(36), and 73(100).

ent-2α, 3α, 10,17-Tetrahydroxy-20-norgibberellane-7,19-dioic Acid 7-Methyl ester 19, 10-Lactone (17-hydroxyGA_{34} methyl ester, CCXVII)

Methyl ester of gibberellin A_{34}, CCV (5mg) was dissolved in a little dry THF and was then treated with 0.5M diborane in THF (ca. 5cm^3; a stream of nitrogen gas was passed through before the addition). The reaction mixture was left standing for 2 hours when analytical TLC showed no starting material. Aqueous 5% sodium hydroxide (ca. 5cm^3) and hydrogen peroxide (30% w/v, 2.5cm^3) were added and the resulting mixture was left standing for 1hour at room temperature. More water (10cm^3) was added and the mixture was extracted with ethyl acetate. The ethyl acetate layer was backwashed with water and evaporated in vacuo to give a gum (5mg). GLC analysis indicated only one product. The TMSi derivative was prepared and the mass spectrum (Fig. 25) run. m/e 598 (21%), 597 (40), 596 (M^+, 100), 581 (1), 462 (5), 301 (6), 289 (10), 288(9), 284 (9), 261 (8), 245 (3), 241 (4), 233 (6), 229 (7), 223(7), 219 (5), 218 (10), 217 (17), 211 (6), 201 (5), 181 (6), 147 (19), 143 (5), 129 (6), 103 (12), 75 (20) and 73 (38).

Epimerisation of the hydroboration product

1. 1st Method

(a) Acetonisation

The 17-hydroxyGA_{34} methyl ester, CCXVII (2mg) (obtained from the

hydroboration of $MeGA_{34}$) was acetonated by dissolving in dry acetone (1 cm^3) containing about 0.001% concentrated sulphuric acid. The mixture was left for about 24 hours (reaction followed by TLC). A small amount of sodium bicarbonate was added to destroy the sulphuric acid and the mixture was evaporated. A little water was added and the mixture was extracted with ethyl acetate. The ethyl acetate layer was backwashed with a little water and then evaporated to give a gum. Analysis of the gum on the GC-MS showed that it was mainly the 2,3-acetonide of 17-hydroxy gibberellin A_{34} methyl ester, CCXX (about 95% by GLC estimation.) m/e 420 (M^+, 6%), 406 (19), 405 (66), 403 (28), 402 (26), 388 (31), 387 (72), 344(16), 312 (35), 283 (28), 256 (28), 241 (35), 239 (56), 228 (28), 233 (100), 180 (25), 179 (31), 171 (28), 155 (22), 95 (16), 71 (19), 57 (26), 55 (26) and 43 (38).

(b) Oxidation of the acetonide

The acetonide, CCXX obtained above, without further purification was oxidised with chromium trioxide-pyridine complex prepared[189] in situ as described below.

A mixture of dry dichloromethane (250µl) and dry pyridine (10µl ≃ 0.12mM) was cooled with stirring to 10°C. Chromium trioxide (6mg ≃ 0.06mM) was added to the mixture once. The stirring was continued at 10°C for five minutes and the mixture was then allowed

to warm slowly (with stirring) to 20°C over about 60 minutes. The crude acetonide (4mg ≈ 0.01mM) in dry dichloromethane (50µl) was added to the complex with stirring. The reaction was allowed to go on for about 15 minutes. The organic layer was decanted, 5% aqueous sodium bicarbonate was added to it and it was extracted with ethyl acetate. The ethyl acetate layer was backwashed with water and evaporated to give a gummy product (3.5mg). Analysis on the GC-MS revealed that the aldehyde, CCXXI was the major product (ca.80% by GLC estimation). m/e 418 (M^+, 9%), 404 (21), 403 (92), 244 (11), 240 (20), 239 (100), 183 (10), and 43 (15).

(c) Epimerisation of the aldehyde [CCXXI] and the reduction of the resulting epimer

The crude aldehyde, CCXXI (2mg) was dissolved in tetrahydrofuran (50µl). 5M aqueous sodium hydroxide (1 cm^3) was added and the mixture was stirred at room temperature overnight. The mixture was acidified and extracted with ethyl acetate. The ethyl acetate layer was evaporated, after backwashing with water to give a gum (ca.2mg). GLC analysis showed that there was one major product (probably CCXXII).

The gummy product (ca.2mg) was dissolved in ethanol (1.5 cm^3) and sodium borohydride (4mg) was added. The mixture was stirred overnight at room temperature. The mixture was neutralised with

dilute hydrochloric acid and extracted with ethyl acetate. The ethyl acetate extract was evaporated (after backwashing with water) to give a gummy product (ca. 2mg). GLC analysis indicated only one major (ca. 70%) component with a longer GLC retention time than the aldehyde, CCXXI.

(d) Deacetonation

The gummy product (ca. 2mg) obtained above was dissolved in methanol (1 cm^3) and a catalytic amount of p-toluenesulphonic acid was added. The mixture was left overnight at room temperature. Ethereal diazomethane was added to destroy the p-toluenesulphonic acid and the mixture was evaporated to give a gum (ca. 2mg). Analysis of the gum on the GC-MS as the trimethylsilyl ether indicated that the alcohol, CCXXIII was the major product.

m/e (of TMSi derivative of CCXXIII, Fig. 26) 598 (23%), 597 (49), 596 (M^+, 100), 581 (9), 462 (6), 301 (6), 289 (9), 288 (16), 284 (8), 261 (6), 245 (13), 241 (4), 233(8), 231 (9), 229 (9), 223 (9), 219 (6), 218 (12), 217 (23), 211 (6), 201 (5), 181(7), 147 (22), 143 (8), 129 (6), 103 (16), 75 (29) and 73 (51).

2. 2nd method

(a) Acetylation of gibberellin A_{34} methyl ester, CCV

Gibberellin A_{34} methyl ester, CCV (2mg) was dissolved in acetic anhydride (1 cm^3) and a catalytic amount of p-toluenesulphonic

acid was added. The mixture was left overnight at room temperature after which analytical TLC showed no starting material. Ethereal diazomethane was added to destroy the excess reagent and the mixture was evaporated to give a gum (ca. 2mg).

(b) Hydroxylation of the 2,3-diacetate of $MeGA_{34}$, CCXXV

The crude 2,3-diacetate of gibberellin A_{34} methyl ester, CCXXV obtained above was dissolved in tetrahydrofuran (1 cm^3) and osmium tetroxide (ca. 2mg) was added. The mixture was stirred overnight. The resulting osmate ester was cleaved in the usual way[175] (sodium metabisulphite 400mg, water 500µl, and pyridine 600µl). On work-up a gummy product (2.8mg) was obtained. Analysis of the product on GC-MS as the trimethylsilyl ether showed that it was mainly (over 95%) the 2,3-diacetate of 16,17-dihydroxy gibberellin A_{34} methyl ester [CCXXVI] and a little monoacetate. m/e (of the TMSi derivative of CCXXVI) 624 (M^+, 1%), 609 (1), 523 (10), 522 (37), 521 (100), 217 (4), 147 (9), 143 (3), 129 (3), 75 (7), 73 (18) and 43 (6).

(c) Dehydration of the 2,3-diacetate of 16,17-dihydroxy $MeGA_{34}$

The crude 2,3-diacetate of 16,17-dihydroxy gibberellin A_{34} methyl ester [CCXXVI] was dissolved in tetrahydrofuran (1 cm^3) containing concentrated sulphuric acid (1-2%). The mixture was refluxed gently for 20 minutes, cooled, neutralised with 5% aqueous

sodium bicarbonate solution and extracted with ethyl acetate. The ethyl acetate layer was evaporated after backwashing with a little water. The gummy product (ca.1mg) was analysed on the GC-MS. The analysis indicated that the aldehyde [CCXXVII] was the major (ca. 60%) product. m/e (of CCXXVII) 462 (M^+, < 1%), 431 (4), 420 (8), 402 (5), 360 (5), 346 (8), 304 (14), 303 (12), 298 (34), 286 (14), 240 (19), 239 (55), 238 (94), 183 (19), 182 (40), 181 (22), 179 (14), 155 (14), 115 (16), 73 (18), 71 (35), 55 (16) and 43 (100).

(d) Reduction and deacetylation

The gummy product (ca. 1mg) obtained above was taken into ethanol (1cm^3) and sodium borohydride (2mg) was added. The mixture was stirred overnight. Acetone (2cm^3) was added to destroy the excess sodium borohydride. Water (1cm^3) was added and the mixture was concentrated (ca. 0.5cm^3). The concentrated mixture was extracted with ethyl acetate. The ethyl acetate layer was backwashed with water and then evaporated to give a gum (ca.1mg).

The gum (ca.1mg) was deacetylated by treating its solution in a little methanol (ca.250µl) with 5% potassium methoxide (ca. 750µl) for about 3 hours. The mixture was neutralized with dilute hydrochloric acid. Water (1.5cm^3) was added and the mixture was concentrated (ca. 1 cm^3). The concentrated mixture was extracted with ethyl acetate. The ethyl acetate layer was evaporated (after

backwashing with water) to give a gummy product (< 1mg). The product was analysed on the GC-MS as the trimetylsilyl derivative. The mass spectrum of the major product was identical with the mass spectrum (Fig. 26) of the trimethylsilyl derivative of the alcohol, CCXXIII.

ent-2α, 3α, 10, 16β-Tetrahydroxy-20-norgibberellane-7,19-dioic Acid 7-Methyl ester 19, 10-Lactone (16α-hydroxyMeGA$_{34}$, CCXXVIII)

3M dilute hydrochloric acid (600µl) was added to a solution of gibberellin A$_{34}$ methyl ester, CCV (1 mg) in methanol (150µl).[193] The mixture was left at room temperature for two days (monitored with TLC). Water (1 cm^3) was added to the mixture and the mixture was extracted with ethyl acetate. The ethyl acetate layer was backwashed with water, and evaporated giving a gummy product (1mg). The product was analysed on the GC-MS as the trimethylsilyl ether derivative. The GC-MS analysis indicated that the 16α-hydroxy gibberellin A$_{34}$ methyl ester [CCXXVIII] was the major (over 90%) product. m/e (of TMSi derivative of CCXXVIII) 597 (44%), 596 (M^+,92), 581 (11), 506 (15), 289 (13), 288 (15), 223 (10), 218 (13), 217 (18), 157 (15), 156 (13), 147 (27), 143 (23), 131 (28), 130 (100), 129 (8), 117 (13), 115 (15), 75 (61) and 73 (90).

APPENDIX

HALO BIOASSAY ON THE ACIDIC ETHYL ACETATE (AE) EXTRACTS OBTAINED FROM 6-DAY OLD AND 2-DAY OLD ADZUKI FRUITS

Introduction

The results that were obtained in the analysis of some of the hormones in the acidic ethyl acetate (AE) extracts obtained from 2-day old and 6-day old New Era fruits showed that the 2-day old fruits were deficient in growth promoting hormones. This indicated that the abscission of the younger fruits might be due to the lack of adequate concentration of growth promoting hormones in these fruits.

The New Era cultivar of cowpea was known[2,6] to exhibit a high degree of abscission of flower buds, flowers and immature fruits.

It was decided to carry out a preliminary study of the hormonal content of another cultivar of cowpea that exhibited a relatively low degree of the abscission problem.

The Adzuki cultivar of cowpea had been reported[2,6] to exhibit a lower degree of the abscission problem than New Era. The Adzuki cultivar was therefore used.

Extracts were obtained from two groups of fruits. These were 6-day old fruits from the lowest raceme and 2-day old fruits from the upper racemes.

Results and Discussion

(a) Halo bioassay on TLC Rf zones of the AE fraction from 6-day old fruits.

The results of the halo bioassay carried out on the extracts of the ten equal TLC Rf zones of the PVP purified AE fraction from 6-day old Adzuki fruits are shown in Table 8 below.

Gibberellin activity was obtained at Rf zones 0.3 - 0.4, 0.4 - 0.5 and 0.5 - 0.6.

TABLE 8

Results obtained in the halo bioassay on TLC Rf zones of the AE extract from 6-day old Adzuki fruits.

TLC Rf Zones	Diameter of halo (in mm) for each half seed											
	1	2	3	4	5	6	7	8	9	10	TOTAL	MEAN
0.0 - 0.1	0	0	0	0	0	0	0	0	0	0	0	0
0.1 - 0.2	0	0	0	0	0	0	0	0	0	0	0	0
0.2 - 0.3	0	0	0	0	0	0	0	0	0	0	0	0
0.3 - 0.4	8	8	10	10	8	9	8	8	7	9	85	9 ± 1
0.4 - 0.5	11	10	11	12	10	10	12	9	10	10	105	11 ± 1
0.5 - 0.6	8	9	10	10	10	12	12	13	12	10	106	11 ± 1
0.6 - 0.7	0	0	0	0	0	0	0	0	0	0	0	0
0.7 - 0.8	0	0	0	0	0	0	0	0	0	0	0	0
0.8 - 0.9	0	0	0	0	0	0	0	0	0	0	0	0
0.9 - 1.0	0	0	0	0	0	0	0	0	0	0	0	0
CONTROL	0	0	0	0	0	0	0	0	0	0	0	0
Standard GA_3 0.5 $\mu g/cm^3$	10	10	12	11	10	9	10	10	9	10	101	10 ± 1

(b) Halo bioassay on TLC Rf zones of the AE fraction from 2-day old fruits.

The results of the halo bioassay carried out on the extracts of ten equal TLC Rf zones of the PVP purified AE extract from 2-day old Adzuki fruits are shown in Table 9 below.

TABLE 9

Results obtained in the halo bioassay on TLC Rf zones of AE extract from 2-day old Adzuki fruits.

TLC Rf Zones	Diameter of the halo for each half-seed (in mm)											
	1	2	3	4	5	6	7	8	9	10	TOTAL	MEAN
0.0 - 0.1	0	0	0	0	0	0	0	0	0	0	0	0
0.1 - 0.2	0	0	0	0	0	0	0	0	0	0	0	0
0.2 - 0.3	0	0	0	0	0	0	0	0	0	0	0	0
0.3 - 0.4	0	0	0	0	0	0	0	0	0	0	0	0
0.4 - 0.5	4	5	6	6	5	5	5	6	5	5	52	5 ± 1
0.5 - 0.6	4	5	5	6	6	4	5	5	5	5	50	5 ± 1
0.6 - 0.7	0	0	0	0	0	0	0	0	0	0	0	0
0.7 - 0.8	0	0	0	0	0	0	0	0	0	0	0	0
0.8 - 0.9	0	0	0	0	0	0	0	0	0	0	0	0
0.9 - 1.0	0	0	0	0	0	0	0	0	0	0	0	0
CONTROL	0	0	0	0	0	0	0	0	0	0	0	0
Standard GA_3 $0.5\mu g/cm^3$	10	10	12	11	10	9	10	10	9	10	101	10 ± 1

Comparison of the results obtained for Adzuki fruits with those obtained for New Era fruits

The results obtained for Adzuki fruits differed from the results obtained for New Era fruits in that no gibberellin could be detected in 2-day old New Era fruits. Thus the 2-day old fruits of Adzuki (a cultivar that exhibits a relatively low degree of the abscission problem) had a detectable amount of gibberellins, whereas the 2-day old fruits of New Era (a cultivar that exhibits a relatively high degree of the abscission problem) had no detectable amount of gibberellins.

This preliminary results therefore support the proposition that the abscission of the immature fruits might be partly due to lack of adequate concentration of growth promoting hormones.

Experimental

The 6-day old Adzuki fruits (50g fresh weight) were extracted according to the procedure used for 6-day old New Era fruits (page 217) giving the acidic ethyl acetate fraction (43 mg).

The 2-day old Adzuki fruits (50g fresh weight) were extracted according to the procedure used for 2-day old New Era fruits (page 215) giving the acidic ethyl acetate fraction (41mg).

The TLC of the PVP purified extracts was carried out as described for the extracts from New Era fruits (page 225) and the halo bioassay was carried out as described in page 227.

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