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THE SYNTHESIS OF [^{3}H]GIBBERELLIN A_3 AND [^{3}H]GIBBERELLIN A_1 BY THE PALLADIUM-CATALYZED ACTIONS OF CARRIER-FREE TRITIUM ON GIBBERELLIN A_3

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Key Word Index—Tritiated GA_3, Tritiated GA_1; tritium exchange reaction, GLC determination of specific activity, selective reduction, carrier-free tritium; stereochemistry of reduction

Abstract—Reaction of gibberellin A_3 (GA_3) with carrier-free tritium gas and 5% palladium on calcium carbonate as catalyst gave a complex mixture of products, several of which were isolated and identified. Three of the purified products are the radioactive forms of naturally occurring gibberellins [^{3}H]GA_3 (**1**), [^{3}H]GA_1 (**2**) and [^{3}H]tetrahydro GA_3 (**4**). Another substance was isolated and tentatively identified as [^{3}H]16,17-dihydro GA_3 (**3**). GLC was used to determine the specific activities of **1** and **2**. [^{3}H]GA_3 likely arises from palladium catalyzed nonspecific exchange of GA_3 alkane hydrogen atoms with tritium. [^{3}H]GA_1 is also exchange labeled but most of its radioactivity is due to tritium addition to the C-1,2 olefinic bond of GA_3.

INTRODUCTION

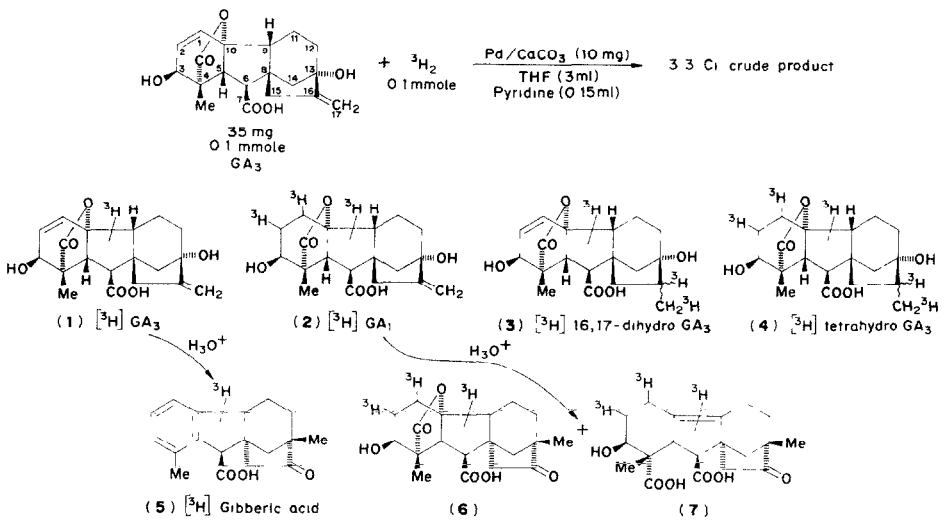
EXTENSIVE investigations have shown that catalytic hydrogenation of GA_3 results in the formation of a mixture of products.[1,2] Some workers have attempted to optimize the reaction conditions for selective reduction of GA_3 to GA_1.[3,4] We previously reported[5] the purification of [^{3}H]GA_1 which had been prepared by the selective reduction procedures of Jones and McCloskey.[3] Subsequently, to obtain [^{3}H]GA_1 of higher specific activity (sp. act.) for studies of gibberellin metabolism, we used the more recently published modification of Pitel and Vining.[4] We found that the resulting product mixture contained compounds which had not been detected previously,[1–5] and which were not separable by our reported methods. We therefore set out to find a new purification scheme and to identify the unknown products.

RESULTS

The reduction of GA_3 with tritium gas was carried out by procedures similar to those reported [4] (Scheme 1). The conditions of this procedure are supposed to optimize the production of [^{3}H]GA_1 and to minimize the formation of side products such as dibasic acids resulting from hydrogenolysis of the lactone ring, and "over-reduced" products resulting from lack of selectivity of tritium addition. Our results show that even under these conditions 26% of the radioactivity in the mixture is in dibasic acids (Fig. 1, zone C). Of the

[1] MULHOLLAND, T. P. C. (1963) *J. Chem. Soc.*, 2606
[2] ALDRIDGE, D. C., GROVE, J. F., MCCLOSKEY, P. and KLYNE, W. (1963) *J. Chem. Soc.*, 2569
[3] JONES, D. F. and MCCLOSKEY, P. (1963) *J. Appl. Chem.* **13**, 324
[4] PITEL, D. W. and VINING, L. C. (1970) *Can. J. Biochem.* **48**, 259
[5] NADEAU, R. and RAPPAPORT, L. (1972) *Phytochemistry* **11**, 1611

total radioactivity in monobasic acids (zone B), 10% is in over-reduced products (compare zones D and E). Moreover, to our surprise, large amounts of $[^3H]GA_3$ were formed. This compound had not previously been reported as a product in this reaction The purification scheme and the results obtained are summarized in Table 1 and Fig. 1. The scheme consists of three TLC operations: *step* 1, separation of monobasic acids (zone B) from dibasic acids (zone C) and other unknown radioactive compounds (zone A); *step* 2, separation of C-16,17 nonreduced compounds (**1** and **2**, zone D) from C-16,17 reduced compounds (**3** and **4**, zone E); *step* 3, separation of C-1,2 nonreduced compounds from C-1,2 reduced compounds (**1** from **2** and **3** from **4**) TLC conditions for these steps are summarized in Table 2



SCHEME 1. OUTLINES THE METHOD OF SYNTHESIS AND SHOWS THE STRUCTURES OF FOUR OF THE COMPOUNDS ISOLATED IN PURE FORM, AND THE STRUCTURES OF THE PRODUCTS FROM ACID HYDROLYSIS OF **1** AND **2**.

TABLE 1 RADIOACTIVE COMPOSITION OF THE CRUDE PRODUCT FROM CATALYTIC REDUCTION OF GA_3 WITH TRITIUM

Component	(CPM in component)/(CPM in mixture) × 100 %
1	9 4
2	37
3	1 5
4	3 6
Dibasic acids	26
Other	22

Percentages are based on the relative amounts of radioactivity recovered in each step Actual recoveries equalled approx 34% of the amounts shown in this table because in each of the three steps of the purification, the recovered radioactivity was approx 70% of the applied radioactivity

Compounds **1**, **2** and **4** were identified by GLC comparison of their *Me* and *MeSi* derivatives with derivatized reference compounds, using SE-30 and QF-1 (Table 3). A special GLC technique was used because the compounds of this study have very high sp act

and it would have been wasteful and impractical to routinely inject onto the GLC columns large enough amounts to detect mass (although this was done in determinations of sp. act., see below). Instead, the radioactive test sample was mixed with an appropriate amount of non-radioactive reference gibberellin and the mixture was derivatized. Using a gas chromatograph equipped with an effluent splitter, half of the effluent was diverted to a flame

TABLE 2 TLC MOBILITIES, OF THE RADIOACTIVE COMPOUNDS ENCOUNTERED IN THIS STUDY

Compound	R_{GA1}, TLC System*			
	a	b	c	d
I	0·9	1·0	0·8	0·9
II	1·0	1·0	1·0	1·0
III	0·9	2·1	0·8	0·9
IV	1·0	2·1	1·0	0·9
V	2·6†	—	—	1·8
VI	1·5	—	—	1·1
VII	2·3	—	—	1·5
VIII	2·6†	—	—	1·6

* *System a* (Used in step 1) Stationary phase, ChromAR, solvent, benzene-HOAc (4 1), 75 min continuous development In this system GA_1 migrated 7 cm from the origin.

System b (Used in step 2). Stationary phase, $AgNO_3$–ChromAR (see Experimental), solvent as in a; 85 min continuous development in which GA_1 migrated 4·2 cm

System c (Used in step 3) Stationary phase, kieselguhr G, solvent, CCl_4–HOAc–H_2O (8 3 5) The aqueous phase was used for equilibrating the plates for 4 hr The organic phase was supplemented with EtOAc to 33% and plates were run for 1 5 hr. GA_1 migrated 9 7 cm

System d Stationary phase, ChromAR; solvent, Et_2O–C_6H_6–HOAc (135·65 10) During 15 min of development the front migrated 15·2 cm and GA_1 7 4 cm

† Radioactivity ran to top edge of strip.

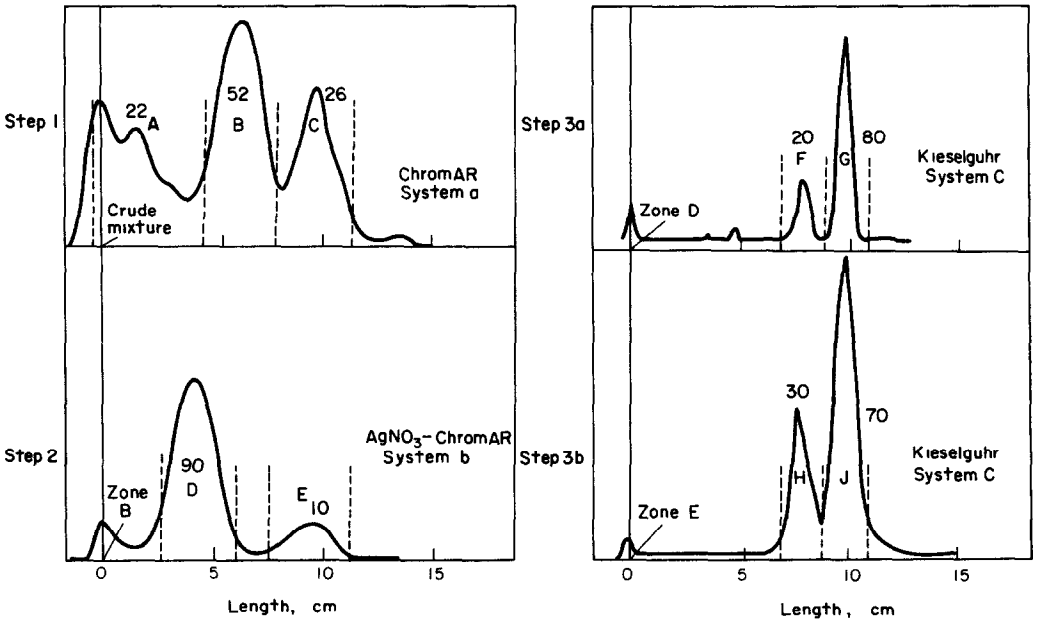


FIG 1 RADIOCHROMATOGRAM TRACES RESULTING FROM THE THREE TLC STEPS OF THE PURIFICATION The numbers near peaks indicate the relative quantities of radioactivity recovered from the zones in each step

ionization detector and the other half to an exit port for collection and subsequent analysis by scintillation counting. By collecting fractions at 0·5 min intervals and plotting the scintillation count data on top of the trace obtained from the flame ionization signal, we could determine whether the radioactive substance corresponded to the "cold" reference gibberellin. For example, GA_3 was combined with radioactivity from zone F (Fig. 2) and this mixture was derivatized (*MeSi*) and injected onto 3% QF-1 (see Fig 2) The results showed a close correspondence between the traces for radioactivity and mass. It should be noted that two peaks were obtained for both the radioactivity and mass traces, a recurrent observation for this GA_3 derivative Freshly prepared samples of GA_3*MeSi* tended to have more of the 7·2 min peak than did samples which had stood for extended periods of time; in all samples, the 9 3 min peak was the dominant one When the 9·3 min peak was collected

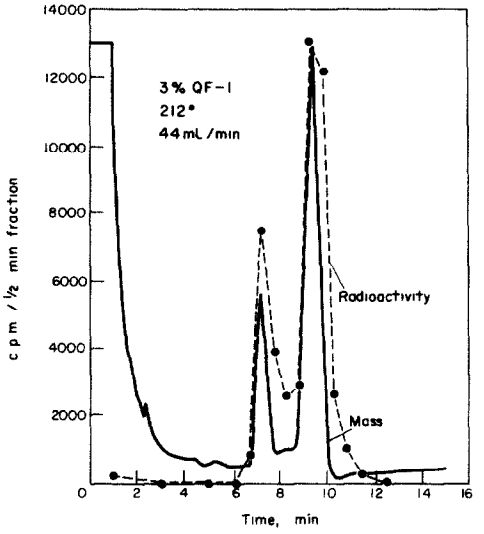


FIG 2 GLC COMPARISON OF AUTHENTIC GA_3 WITH [^{3}H]GA_3 PRODUCED IN THE $^3H_2$/Pd REACTION (Details in text)

and re-injected a new peak, distinct from either of the previously observed peaks, was observed at 7·7 min (data not shown). Several GA_3 samples from different sources gave the same results. Thus GA_3*MeSi* appears to be unstable under the GLC conditions we used. The qualitative method described above was also used to identify the compounds in fractions **2** and **4**. In addition, acid hydrolysis of **1** and **2** gave products whose derivatives had GLC properties corresponding to the derivatized acid-hydrolysates of the appropriate reference compounds (see Fig. 1 and Table 3) However, **3**, which is a minor component of the crude mixture, has not previously been reported or isolated and a reference sample was not available for comparison Thus, the structure assigned to **3** is tentative and is based on the following observations: (1) In TLC systems b and c the mobility ratios **1** **2** and **3**:**4** are the same, as would be expected if **3** bears the same structural relationship to **4** as **1** does to **2**; (2) **3**, like **4**, should be a pair of epimers, a prediction which was confirmed by GLC of **4**-*Me* on QF-1 (see Table 3); (3) Acid hydrolysis of **3** gave a product (**8**) with TLC and GLC properties similar to gibberic acid, indicating that **3** may well have the same ring-A structure as does GA_3.

TABLE 3 GLC RETENTION TIMES OF COMPOUNDS IN THIS STUDY*

	Liquid phase			
	2% SE-30		3% QF-1	
	Me	*MeSi*	*Me*	*MeSi*
1 and GA_3	9·5 b	10·7	24·2 b	7·2, 9·3
2 and GA_1	9·2	9·9	19·7	8·1
3	9·5 b	ND	20·3, 22·4	ND
4	9·5 b	10·3	20·5, 22·9	8·6
Tetrahydro GA_3	10·0	10·3	22·9	8·6
Epitetrahydro GA_3	9·3	10·3	20·5	8·4
5 and gibberic acid	2·5 [8·4]	—	2·6 [12·4]	—
6 and GA_1-ketone	9·0	9·0	31·0	21·2
7	7·1	6·7	11·0	6·2
8	3·0 [11·7]	ND	2·4 [10·3]	[5·8]

* 3% QF-1 and 2% SE-30, both coated on 100–120 mesh GAS-CHROM Q, were used in stainless steel columns measuring 83 cm × 3 mm. Conditions: oven temp (212°), carrier gas flow (N_2 44 ml/min) apply to all the R_t's in this table except those in brackets, in which the oven temp was 180° and all other conditions remained the same

Injector temp = 225°, detector temp = 230°; b = broad peak. ND = not determined

Detection of small amounts of **1** in samples containing predominantly **2**, using the effluent splitter method, was impractical because of the similar retention times of these compounds. Therefore, we developed a method which takes advantage of the fact that the end product of GA_3 acid hydrolysis (**5**, gibberic acid) has GLC properties quite distinct from the acid hydrolysis products[6] of **2** (**6** and **7**, Fig. 1). First, approximately 1 million cpm of the test sample was combined with 20 μg of reference GA_1 and and the mixture was heated in a boiling water bath for 30 min. The product was isolated by extraction into ethyl acetate and was subsequently derivatized (*MeSi*) and taken up in 20 μl of ethyl acetate.

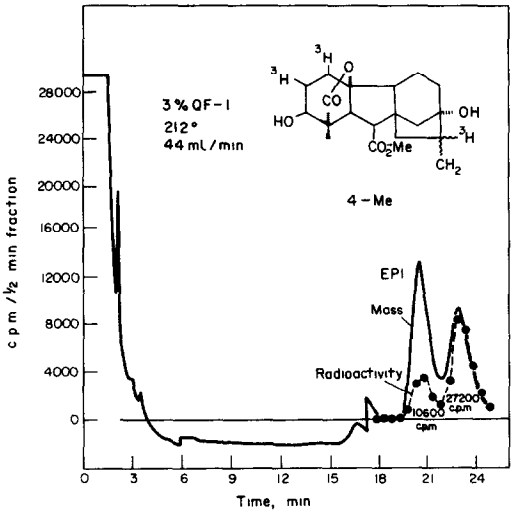


FIG 3 GLC DETERMINATION OF THE EPIMER RATIO IN [^{3}H]TETRAHYDRO GA_3 (Details in text)

[6] BRIAN, P. W., GROVE, J. F. and MACMILLAN, J. (1960) *Fortschr. Chem. org. Naturstoffe* **18**, 350

One μl (approximately 1 μg, 50000 cpm) of the hydrolysate-*MeSi* was injected onto an SE-30 column and three zones, corresponding to the retention times of **5**-*Me*, **6**-*MeSi* and **7**-*MeSi*, were collected. Since reference GA_1 was added prior to the hydrolysis, the *MeSi* derivatives of **6** and **7** corresponded to mass peaks and were readily detected and collected. When conditions were adjusted such that the retention time of **6**-*MeSi* was 9·0 min, the retention time of **5**-*Me* was 2·5 min and it was easily obtained in a fraction collected between 2 and 3 min. Samples of **2** which were free of **1** gave zero cpm (above background) in the 2–3 min collection. In impure samples, the percentage of **1** was calculated as (cpm in the 2–3 min collection/cpm collected under peaks for **6**-*MeSi* and **7**-*MeSi*) × 100

The ratio of the epimers of **4** was determined by GLC, using reference samples. The epimers, whose absolute configurations are unknown, are distinguishable by GLC of the methyl esters on QF-1. The epimer with the shorter retention time is called *epi*-tetrahydro GA_3, and the other simply tetrahydro GA_3.[7] Recently, tetrahydro GA_3 was shown to be a naturally occurring gibberellin.[8] By GLC of **4**-*Me* (Fig. 3), we showed that the [^{3}H]tetrahydro GA_3:[^{3}H]*epi*-tetrahydro GA_3 ratio was 2·6:1.

The sp. act. of both **1** and **2** were measured by determining the activity necessary to give the same-sized peak as produced by a known mass of the corresponding reference compound in GLC (see Experimental).

DISCUSSION

Surprisingly, radioactivity due to [^{3}H]GA_3 comprised 9·4% of the crude reaction mixture (vs 37% for [^{3}H]GA_1) and had high sp. act. (13 Ci/mmole vs 43 Ci/mmole for [^{3}H]GA_1). Apparently, the reaction conditions for this preparation permitted considerable tritium exchange with alkane hydrogen atoms of the GA molecules. The mechanism of this exchange reaction may parallel results obtained in heterogeneous–catalytic exchange reactions between deuterium and alkanes.[9] In such reactions, exchange is maximal when the catalyst is palladium and is least when it is rhodium. It should be noted that the kind of exchange reaction mentioned above is distinct from the Wilzbach gas–exposure reaction.[10] Moreover, the Wilzbach method gives products of much lower sp. act. than exists in our [^{3}H]GA_3. It should be noted that neither the mechanism for the production of the latter compound from GA_3 nor the position of label has been determined. Since **1** is exchange labeled to the extent of 13 Ci/mmol it seems likely that all of the other [^{3}H]GA molecules in the product mixture are equally labeled. Hence, we assume that **2** (sp. act. = 43 Ci/mmol) is randomly labeled to the extent of 13 Ci/mmol and specifically labeled (C-1,2) to the extent of 30 Ci/mmol. We have not measured the sp. act. of **3** or **4** but, by similar reasoning to the above, we expect **3** has sp. act. = 43, and **4**, sp. act. = 73 Ci/mmole (2 × 30 + 13). Because the tritium used in this preparation was carrier-free (59 Ci/mmol), it might be expected that the sp. act. of **2** should be at least as great, and perhaps greater due to the exchange reaction. However, the conditions of the reaction apparently promote such rapid exchange of tritium, not only with GA molecules but also with solvent molecules, that the tritium gas quickly becomes diluted with hydrogen.

[7] GASKIN, P., MACMILLAN, J., GANGULY, S. N., SANYAL, T., SIRCAR, P. K. and SIRCAR, S. M. (1972) *Chem. & Ind.*, 424.
[8] GANGULY, S. N., SANYAL, T., SIRCAR, P. K. and SIRCAR, S. M. (1970) *Chem. & Ind.*, 832.
[9] BURWELL, R. L. (1972) *Catalysis Rev.* **7**, 25.
[10] WILZBACH, K. E. (1962) *Tritium in the Physical and Biological Sciences, Symp. Intern. At. Energy*, **2**, 3.

Since the epimers of **4** were formed in a ratio of 2·6:1 (Fig. 3) addition of tritium to the C-16,17 bond is moderately stereoselective under these reaction conditions. Work in progress in our laboratory, on the enzymic hydroxylation of [^{3}H]GA_1 in the C-2(β) position to produce [^{3}H]GA_8, has provided an insight into the configuration of the tritium atoms at C-1,2 of [^{3}H]GA_1. In this work, the formation of tritiated water is used as an assay to measure [^{3}H]GA_8 formation. Calculations based on the amount of tritiated water formed per amount of [^{3}H]GA_8 formed, indicate that virtually all of the [^{3}H]GA_1 has its C-1,2 tritium atoms in a β configuration.

The radioactive GAs produced are used in studies on metabolism of GAs in plants and it is therefore essential to achieve high purity. For example, in one of our unpublished experiments, we used what we thought to be pure [^{3}H]GA_1 to measure metabolism of the hormone in barley seeds. A "metabolite" appeared in a control portion of the experiment which was later shown to be a derivative of [^{3}H]GA_3 that had arisen during autoclaving. Under the same autoclaving conditions, **2** is stable. GA_3, whether in the "cold" or "hot" form, is an inevitable impurity in [^{3}H]GA_1 synthesized from GA_3, and special steps must be taken to eliminate it during purification. For this purpose, the kieselguhr method[11] (steps 3a, b) is adequate, but clean separations such as are indicated in Fig. 2 are difficult to achieve consistently. Other workers may wish to substitute a different way to separate **1** from **2** in the final step of the purification.

Although the reaction conditions for the preparation were meant to duplicate those described by Pitel and Vining,[4] certain differences were bound to occur. We used carrier-free tritium, whereas Pitel and Vining used a mixture of hydrogen and tritium. The quantity of starting GA_3 was much smaller in our reaction than in theirs (35 mg vs 2 g) and thus there was greater probability of error in determining the endpoint or our reaction. Perhaps this accounts for differences in the results of our work and that of others who prepared [^{3}H]GA_1.

Table 1 gives the percentage of each component found in the mixture but not actual recovered yields. In each of the three TLC steps the recovery of radioactivity is approximately 70%. Thus, in a typical purification run which started with $1{\cdot}5 \times 10^{11}$ cpm the overall recovered yields where approximately $4{\cdot}7 \times 10^9$ cpm of pure [^{3}H]GA_3 (3·2%) and $1{\cdot}9 \times 10^{10}$ cpm of [^{3}H]GA_1 (12·6%). It is evident, therefore, that the overall percentage yields are somewhat low but that the cpm's attainable from a single purification procedure are nevertheless quite high. This outcome, of course, is due to the very high sp. act. of the products.

For those who may wish to prepare [^{3}H]GA_1 for biological experiments, the paramount concern must be with obtaining pure hormone. For this reason it is desirable to optimize reaction conditions for the production of [^{3}H]GA_1. However, no matter which procedure is used the reaction mixture will contain at least small amounts of all the possible side-products and these will have to be eliminated. Fortunately, GA_3 is readily available, and carrier-free tritium is inexpensive. These results hopefully provide purification procedures which will help others avoid the pitfalls inherent in the selective reduction procedure.

EXPERIMENTAL

TLC methods Development solvents are listed in Table 2 In all of the TLC operations, ChromAR strips (Mallinckrodt Chemical Works) measuring 5 × 20 cm were used and sample was applied 3 cm from the bottom edge

[11] KAGAWA, T., FUKINBARA, T. and SUMIKI, Y (1963) *Agric Biol Chem* **27**, 598

$AgNO_3$-impregnated ChromAR was prepared by dipping 5 × 20 cm strips in aq 5% $AgNO_3$ solution, placing these strips on glass, and drying them at 110° for 0 5 hr A special method was used to "elute" radioactivity from $AgNO_3$–ChromAR A regular 5 × 20 cm piece of ChromAR was cut into 2 pieces, measuring 5 × 8 cm and 5 × 12 cm, and both pieces were placed on top of a 5 × 20 cm glass plate A space between the 2 pieces was left such that a radioactive zone (e.g zone D or E) could be inserted between them, overlapping each by ~2 mm Then a 5 × 20 cm glass plate was placed on top, and the "sandwich" with the top piece of ChromAR protruding a few cm, was held together with rubber bands The sandwich was placed in a chromatographic developing tank containing solvent, EtOAc–$CHCl_3$–HOAc (15 5·1) for 30 min As the solvent travelled upward it moved the radioactive material from the $AgNO_3$–ChromAR section onto regular ChromAR Radiochromatogram scanning was used to detect the new radioactive zone, now free of $AgNO_3$

In scanning, peaks which contained more than 5 × 10^8 cpm presented a special problem because of instrument limitations The problem was solved by making an imprint of the "hot" strip by gently pressing it together with a ChromAR strip of the same size, and scanning the imprint This method had to be used in each step

Continuous development of ChromAR strips was conducted in a short developing tank (22 5 cm), equipped with a Plexiglass lid with slits (5 5 × 0 7 cm) The strips was hung from between 2 small glass rods (7 cm long × 3 mm dia.) held together by a small stainless steel spring clip The strip was then suspended so that it dipped into the solvent to a depth of 1 cm Kieselguhr plates (5 × 20 cm) were 0 5 mm in thickness Radioactive materials were eluted from ChromAR and kieselguhr by extraction with 50 50 MeOH–EtOAc

GLC The methods used are similar to those reported by Cavell *et al*[12] Details on packings and conditions are shown in Table 3 and special methods are described in Results Methylated derivatives were prepared using CH_2N_2 To prepare *MeSi* derivatives *Me's* were further treated with Sil Prep (hexamethyldisilazane trimethylchlorosilane–pyridine, 3 1 9)

Reaction of GA_3 with tritium The following protocol (furnished by chemists at New England Nuclear, Boston, Mass., who conducted the reaction) was used A mixture of GA_3 (35 mg), tetrahydrofuran (3 ml), pyridine (0 15 ml), 5% Pd on $CaAO_3$ (10 mg; prereduced with H_2) was stirred in an atmosphere of carrier-free $^3H_2$ gas (sp act 59 Ci/mmol) until 2 5 ml of gas was absorbed The mixture was taken to dryness under vacuum Labile $^3H_2$ was removed two times with MeOH The catalyst was removed by filtration through a millipore filter The product (3 3 Ci) was dissolved in MeOH (2 ml) and EtOAc (8 ml) One third of the product was dissolved in 500 ml of 95% EtOH, providing a stock soln containing 1 5 × 10^6 cpm/μl

Purification Step 1 100 Ml of stock soln, containing 1 5 × 10^{11} cpm was dried under vacuum The radioactive residue weighing 2 4 mg was dissolved in *ca* 100 μl of MeOH, streaked onto a 5 × 20 cm strip of ChromAR, and developed continuously (system a) for 75 min After scanning, the strip was cut into three sections, as shown in Fig 2. Elution gave zone A, 2 3 × 10^{10} cpm, zone B, 5 5 × 10^{10} cpm, zone C, 2 7 × 10^{10} cpm

Step 2 5 0 × 10^{10} cpm of zone B (above) was brought to dryness under a stream of N_2 at 50°, streaked onto a 5 × 20 cm piece of $AgNO_3$ ChromAR and developed in system b (see Table 2) Elution (by the special technique described under *TLC Methods*) gave zone D, 2 9 × 10^{10} cpm, zone E, 3 3 × 10^9 cpm

Step 3 2 0 × 10^{10} cpm of zone D was applied in a single narrow streak near the bottom of a 5 × 20 cm kieselguhr G plate which was developed in system c Isolated were zones F (pure **1**), 2 8 × 10^9 cpm and G (pure **2**), 1 1 × 10^{10} cpm Similar treatment of zone E, 3 2 × 10^9 cpm, in system c gave zone H, 6 7 × 10^8 cpm and zone J, 2 4 × 10^9 cpm

Sp act of [^{3}H]*GA*$_3$ (**1**) To 5 0 μg of cold GA_3 in 2 ml of MeOH was added 6 0 × 10^5 cpm of pure [^{3}H]GA_3 thus producing a reference [^{3}H]GA_3 sample of known sp act (1 2 × 10^5 cpm/μg) To another 2 0 ml of MeOH was added 1 2 × 10^8 cpm of pure [^{3}H]GA_3 producing what is referred to below as the "hot" [^{3}H]GA_3 sample Both solutions were treated with CH_2N_2, dried with N_2, and purified by TLC on ChromAR (solvent = Et_2O–C_6H_6, 2 1) On radiochromatogram scanning, both lots showed a main peak (R_f 0 70) and a minor peak at the origin Recoveries of radioactivity for the samples were "hot" sample, 8 5 × 10^7 cpm, 71%, reference sample, 5 0 × 10^5 cpm, 83% Each sample was dried under N_2, treated with 100 μl of Sil Prep for 1 hr at 25° dried again with N_2, and then combined with ~50 μl of toluene An SE-30 column was used with conditions the same as given in Table 3 except for a higher oven temp (225°) in order to obtain a sharp GA_3*MeSi* peak at short retention time (7 min) A series of 5 × 2 μl injections of the reference sample gave GA_3 *MeSi* peaks measuring 82 to 87 mm in height (average = 84 mm) 3 × 2 μl portions of the reference sample were scintillation counted, giving an average value of 1 7 × 10^4 cpm Thus we calculated that the mass of GA_3*MeSi* which gave the 84 mm high peak was 0·14 μg We then proceeded to inject 2 0 μl portions of the "hot" **1**-*MeSi* sample, attempting to produce peaks whose heights were close to 84 mm By trial and error and by adjusting the volume containing the sample we soon were able to make a series of 4 × 2 μl injections of the "hot" [^{3}H]GA_3 sample which gave peaks ranging in height from 85 to 88 mm, 3 × 2 μl portions of the "hot" sample were scintillation counted, giving an average value of 4 2 × 10^6 cpm/2 μl Hence the sp act of **1** is 4 2 × 10^6 cpm/0 14 μg, this is equivalent to 13 Ci/mmole The sp act of **2**, determined by the same method, was 43 Ci/mmole

High voltage paper electrophoresis Radioactivity from zone C (5 × 10^5 cpm) and a similar amount of **2** were applied as 5 cm long streaks to Whatman 3MM paper (basic wt = 185 g/m^2, 0 33 mm thickness, med flow rate, smooth surface). A high voltage electrophoresis instrument (Savant, Model FP22A) was used Conditions of elec-

[12] CAVELL, B D MACMILLAN J, PRYCE, R J and SHEPPARD, A C (1967) *Phytochemistry* **6**, 867

trophoresis were: buffer, pyridine–HOAc–H_2O (100·15·900) adjusted to pH 6, 1500 V, 1·5 hr Scanning was used to detect radioactivity. Under these conditions, radioactivity from zone C was evidenced as a single peak centered 12 5 cm from the origin on the anode side; **2** migrated a distance of 9·9 cm and a DNP-alanine marker migrated 13·2 cm.

Hydrolysis of [^{3}H]GA samples The sample was brought to dryness in a screw cap (Teflon) test tube (10 cm × 13 mm) and 1·0 ml of 1 N HCl was added The tube was tightly capped and placed in a 400 ml beaker containing 200 ml of boiling H_2O After 30 min the tube was removed, cooled, and the contents were extracted with 3 × 2 ml of EtOAc The extract was shaken with anh. Na_2SO_4 and passed through a small cotton plug and washed with 50: 50 EtOAc-MeOH

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