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HPLC-BASED METHODS FOR THE IDENTIFICATION OF GIBBERELLIN CONJUGATES: METABOLISM OF [^{3}H]GIBBERELLIN A_4 IN SEEDLINGS OF *PHASEOLUS COCCINEUS*

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Key Word Index—*Phaseolus coccineus*; Leguminosae; runner bean; metabolism; HPLC; gibberellins; gibberellin conjugates.

Abstract—A series of chromatographic and derivatization techniques has been developed for the identification of radiolabelled gibberellin (GA) conjugates. The methods are based on reversed-phase HPLC, gel permeation chromatography, anion-exchange chromatography, enzymatic hydrolysis and transesterification of conjugates, and derivatization of free GAs to methoxycoumaryl esters. The procedures have been used to identify GA_4-glucosyl ester, GA_4-3-*O*-glucoside, a GA_{34}-*O*-glucoside and GA_8-2-*O*-glucoside, in addition to GA_1 and GA_8, as products of [1,2-^{3}H]GA_4 metabolism in shoots of light-grown *Phaseolus coccineus* seedlings.

INTRODUCTION

Endogenous plant growth substances such as ABA, IAA, GAs and cytokinins are often covalently linked to other small molecules. In the case of GAs, these so-called 'conjugates' are usually 2β- or 3β-*O*-β-D-glucosyl ethers (*O*-glucosides) or β-D-glucosyl esters [1]. When tested in GA bioassays, biological activities of GA conjugates range from 0 to 100% of that of the corresponding free GA. Available evidence suggests that GA conjugates are inactive *per se* and that their biological activity reflects the degree of hydrolysis to the parent GA [2, 3]. If hydrolysis of conjugates is a normal part of GA metabolism *in vivo*, GA glucosyl ethers and esters may be compounds of major physiological importance since the size of GA pools could be controlled by reversible conversion of free GAs to GA conjugates. The varying susceptibility of individual GA-3β-*O*-glucosides to cellulase treatment *in vitro* [4, 5] suggests that hydrolysis of these conjugates *in vivo* may involve a similarly high degree of enzyme specificity. Studies on the *in vitro* synthesis of GA_3-3-*O*-glucoside (**9**) have also provided some evidence of enzyme specificity in conjugate synthesis [6] which would be in keeping with a regulatory role in GA metabolism.

Gibberellin conjugates accumulate in high concentrations in maturing seed [1] and there are indications that during germination they are reconverted to free GAs [7] which may be involved in the promotion of the early stages of seedling growth. Alternatively, the production of GA conjugates in developing seed may be associated with compartmentation or transport processes prior to degradation. Some mechanism of this type is needed to explain the function of conjugates such as the 2β-*O*-glucosides of GA_8 (**4**), GA_{29} (**6**) and GA_{34} (**3**) which yield biologically inactive aglycones after hydrolysis [2].

Identification of GA conjugates is not straightforward. Trimethylsilyl derivatives are relatively non-volatile, with many having M_rs of *ca* 1000, and thus are not ideally suited for GC/MS analysis. An alternative strategy is to use permethylated derivatives of GA conjugates for GC/MS analysis. Although this approach has yielded satisfactory mass spectra with many GA-glucosyl ethers [8, 9], molecular ions are often not seen, and the most abundant fragments are from the permethylated sugar rather than the conjugate or the aglycone. Thus, in many instances, information on the nature of the conjugate is limited. Furthermore, glucosyl esters cannot be permethylated successfully, as the derivatization procedure causes hydrolysis of the glycosidic linkage [Schneider, unpublished data].

This paper reports on the development of a series of chromatographic and derivatization procedures for the analysis of free and conjugated GAs. These techniques have been used to identify metabolites formed from [^{3}H]GA_4 applied to the epicotyl of light-grown seedlings of *Phaseolus coccineus* L. These seedlings are known to contain GA_1 (**2**), GA_4 (**1**), GA_5 (**7**) and GA_{20} (**5**) [10] all of which, when supplied exogenously, are partially converted to conjugate-like compounds [11–13].

RESULTS AND DISCUSSION

Seven-day-old seedlings of *P. coccineus* were injected with [1,2-^{3}H]GA_4 and extracted after 4 hr. Aliquots of the crude ethyl acetate and butanol fractions were analysed by reversed-phase HPLC–RC. This revealed ten major radioactive compounds, in addition to unmetabolized [^{3}H]GA_4 (peak N) which constituted 31% of the recovered radioactivity (Fig. 1). Most of the metabolites were present in both the ethyl acetate and butanol

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Abbreviations: GA_n, gibberellin A_n; GPC, gel permeation chromatography; CE, methoxycoumaryl ester.

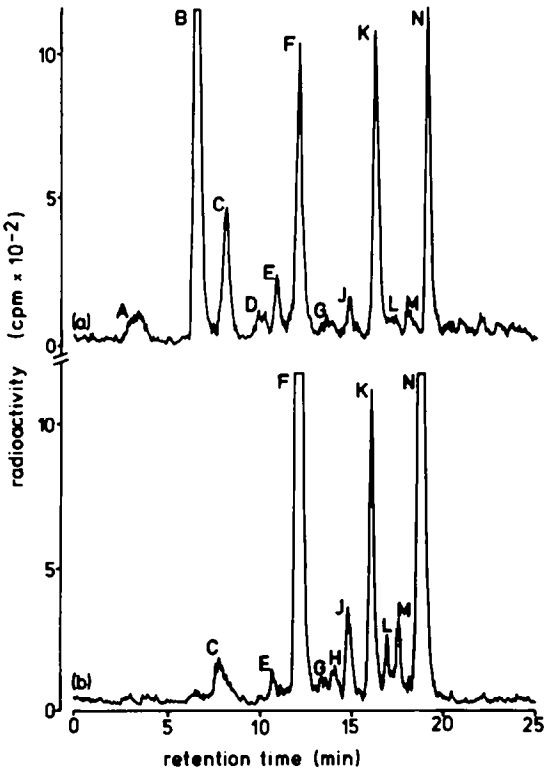


Fig. 1. HPLC profiles of metabolites of [^{3}H]GA_4 fed to epicotyls of *P. coccineus* seedlings. (a) Butanol extract; (b) ethyl acetate extract. Detector: on-line radioactivity monitor. Solvent program 1.

fractions, indicating incomplete separation by the partitioning procedure.

In order to obtain further information on the metabolites, the ethyl acetate and butanol fractions were individually subjected to preparative gel permeation chromatography (GPC), which separated GA conjugates from free GAs. There was also a partial separation of the free GAs according to M_r. Each of the GA-conjugate and free-GA fractions from GPC was then separated into single radioactive peaks by preparative HPLC. The individual components were examined, along with appropriate authentic standards, by analytical HPLC. The data obtained gave indications of the identity of some of the metabolites, but further analysis was needed before firm conclusions could be drawn.

High molecular weight fraction

Putative GA conjugates were analysed by the following procedures: (a) anion exchange chromatography on DEAE-Sephadex A-25 which differentiates between (acidic) glucosyl ethers and (neutral) glucosyl esters; (b) transesterification which converts glucosyl esters to methyl esters whereas glucosyl ethers do not react; (c) cellulase hydrolysis which releases the parent free GA from both ether and ester conjugates; (d) derivatization of cellulase hydrolysis products to methoxycoumaryl esters. After each of these steps, products, along with GA standards, were examined by HPLC. The data obtained, which are summarized in Table 1, indicated that at least four of the peaks shown in Fig. 1 were GA conjugates.

Peak B was acidic and had the same HPLC retention time as GA_8-2-glucoside. In addition, it was not transesterified by sodium methoxide, indicating that it was a glucosyl ether and not a glucosyl ester. Cellulase hydrolysis released a compound co-chromatographing with GA_8 and, after derivatization to the methoxycoumaryl ester, with GA_8-CE. Peak B was thus identified as GA_8-2-*O*-glucoside (**12**).

Peak K did not chromatograph with any of the available GA conjugate standards. However, cellulase hydrolysis released a compound with the HPLC retention time of GA_{34}. This conjugate was deemed to be a glucosyl ether and not a glucosyl ester on the basis of its acidic properties on DEAE-Sephadex and the absence of conversion by the transesterification reagent. Peak K was therefore identified as an *O*-glucoside of GA_{34} (**11**). The position of the glucosyl moiety on the GA molecule could not be ascertained through lack of authentic GA_{34}-glucoside standards. It is interesting to note that a compound with some of the expected GC/MS characteristics of a GA_{34}-glucoside has been detected in extracts from *P. coccineus* seed [8].

Peak L had the same HPLC retention time as GA_4-glucosyl ester. The metabolite was neutral on DEAE-Sephadex and could be transesterified to a compound co-chromatographing with GA_4-methyl ester, thus confirming the ester linkage. Cellulase hydrolysis released a compound with the HPLC retention time of GA_4 and, after derivatization with bromomethylmethoxycoumarin, yielded a compound co-chromatographing with GA_4-CE. Peak L is therefore GA_4-glucosyl ester (**8**).

Peak M had the same HPLC retention time as GA_4-3-*O*-glucoside. It was deemed a glucosyl ether on the basis of acidic properties on DEAE-Sephadex and lack of effect of the transesterification reagent. Cellulase hydrolysis released a compound which co-chromatographed with GA_4 and, after derivatization, with GA_4-CE. Peak M was thus identified as GA_4-3-*O*-glucoside (**10**).

The remaining conjugate-like compounds were not identified. Peak J, thought to be a conjugate on the basis of GPC retention properties, was unstable and was not recovered after preparative HPLC of the conjugate fractions. A small proportion (2%) of the total radioactivity was associated with compounds which, according to the GPC elution profile, have M_rs higher than GA-monoglycoside conjugates. HPLC showed that most of this very high M_r radioactivity was peak D (Fig. 1), but there was insufficient material for further analysis. There was no evidence for the accumulation of GA_1 conjugates, although they were detected as metabolites of [^{3}H]GA_4 in previous studies with *P. coccineus* seedlings [12, 13].

Low molecular weight fraction

Of the free GA fractions, Peaks C and F were thought to be GA_8 (**4**) and GA_1 (**2**), respectively, on the basis of GPC and HPLC retention properties. These identifications were supported after derivatization of these metabolites to methoxycoumaryl esters followed by co-chromatography with authentic compounds on isocratic HPLC (Table 2).

The remaining low M_r compounds were not identified. Peak A, eluting at the void volume, was not seen after GPC, and is therefore assumed to represent highly polar breakdown products possibly including ^3H_2O. The other unidentified peaks (E, G and H) all eluted in the free GA

Table 1. Properties of metabolites detected in high M_r fraction from GPC

Substance	% of total recovered radioactivity	DEAE-Sephadex (acidic/neutral)	Isocratic HPLC (R_t, min)			
			Underivatized	Transesterified	After cellulase hydrolysis	
					Underivatized	Methoxycoumaryl
Peak B	3.2	Acidic	10.6[1]*	n.r.	6.5[2]	7.8[3]
GA_8-2-*O*-glucoside			10.6[1]			
GA_8					6.5[2]	7.8[3]
Peak K	11.1	Acidic	6.5[3]	n.r.	8.4[3]	8.8[5]
GA_{34}					8.4[3]	8.8[5]
Peak L	2.0	Neutral	7.3[3]	11.1[4]	12.4[3]	11.4[3]
Peak M	2.9	Acidic	9.8[3]	n.r.	12.3[3]	11.5[5]
GA_4-glucosyl ester			7.3[3]			
GA_4-3-*O*-glucoside			9.8[3]			
GA_4-Me				11.2[4]		
GA_4					12.3[3]	11.4[5]

Elution volume: 340–365 ml. n.r. = no reaction.
*HPLC conditions 1 = 20% methanol, 2 = 30%, 3 = 60%, 4 = 70%, 5 = 75%.

Table 2. Properties of metabolites detected in low-M_r fractions from GPC

Substance	% of total recovered radioactivity	GPC elution vol. (ml)	Isocratic HPLC (R_t, min)	
			Underivatized	Methoxycoumaryl ester
Peak C	2.9	380	14.4[1]*	7.8[3]
GA_8			14.5[1]	7.8[3]
Peak E	1.4	400	6.3[2]	
Peak F	39.0	405	8.3[2]	10.9[3]
GA_1			8.4[2]	10.9[3]
Peak G	0.5	420		
Peak H	1.3	420		
Peak N	31.0	430	12.2[3]	11.5[4]
GA_4			12.2[3]	11.4[4]

Elution volume: 370–440 ml.
*HPLC conditions: 1 = 20% methanol, 2 = 40%, 3 = 60%, 4 = 75%.

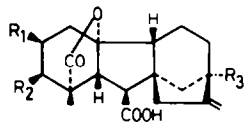
fraction on GPC. These unknown compounds may be catabolic products of the free GAs such as GA_8-catabolite. Despite the relatively large amount of GA_{34}-glucoside (peak K) that accumulated, no free GA_{34} (3) was detected.

The probable metabolic relationships of the compounds identified are illustrated in Fig. 2. With the exception of GA_{34} and conjugates of GA_1, most of the predictable GA_4 metabolites were detected. The formation of GA_1 from GA_4 and the conversion of GA_1 to GA_8-2-glucoside via GA_8 are reactions known to occur in a wide range of tissues [see 1] including seedlings of *P. coccineus* [12–14]. Indications of the formation of GA_4-glucosyl ester as a minor metabolite from [^{3}H]GA_4 have previously been noted in seedlings of *P. coccineus* [11] and in maturing seeds of *P. vulgaris* [15].

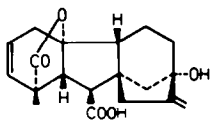
The substrate dose in the current experiment was 590 pmol GA per plant, which is about 100-fold in excess of HPLC-radioimmunoassay estimates of endogenous C_{19}-GA levels in shoots of *P. coccineus* [Turnbull, unpublished data]. In a previous study, with a range of [^{3}H]GA_4 doses up to 15 nmol per seedling, the relative amounts of metabolites formed were similar [12]. The seedlings therefore have a high capacity for metabolizing GA_4, indicating at least the potential for a high rate of GA turnover.

Conversion of [^{3}H]GA_4 to several conjugates, including GA_4-glucosyl ester and GA_4-3-glucoside, has been reported for suspension cultures of anise and carrot [16, 17]. However, identification was based largely on gradient HPLC separations in which, in the absence of an on-line radioactivity monitor, successive fractions were collected to determine the distribution of radioactivity in the eluate. As a consequence, HPLC resolution was significantly reduced and comparison of the retention properties of metabolites and standards was imprecise. The only other evidence provided was hydrolysis of the purported conjugates to yield the parent free GAs which were similarly identified by approximate HPLC retention times. This analytical approach has also indicated the formation of GA_{34}-glucoside from [^{3}H]GA_4 in *Pseudotsuga menziesii* shoots [18].

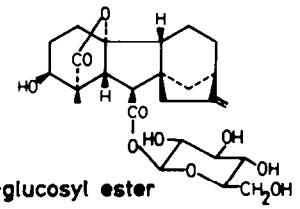
Despite being relatively elaborate, the techniques reported in this paper are currently one of the more practical ways of analysing trace amounts of radiolabelled GAs and GA conjugates. The methods yield extensive information about the properties of these compounds by exploiting differences in M_r, polarity, charge and the nature of the



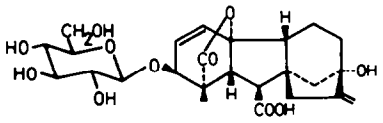
	R_1	R_2	R_3	
(1)	H	OH	H	GA_4
(2)	H	OH	OH	GA_1
(3)	OH	OH	H	GA_{34}
(4)	OH	OH	OH	GA_8
(5)	H	H	OH	GA_{20}
(6)	OH	H	OH	GA_{29}



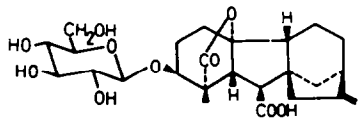
(7) GA_5



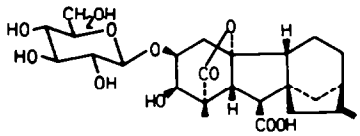
(8) GA_4-glucosyl ester



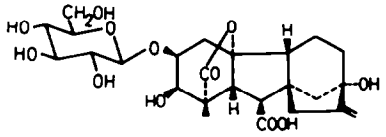
(9) GA_3-3-O-glucoside



(10) GA_4-3-O-glucoside



(11) GA_{34}-O-glucoside



(12) GA_8-2-O-glucoside

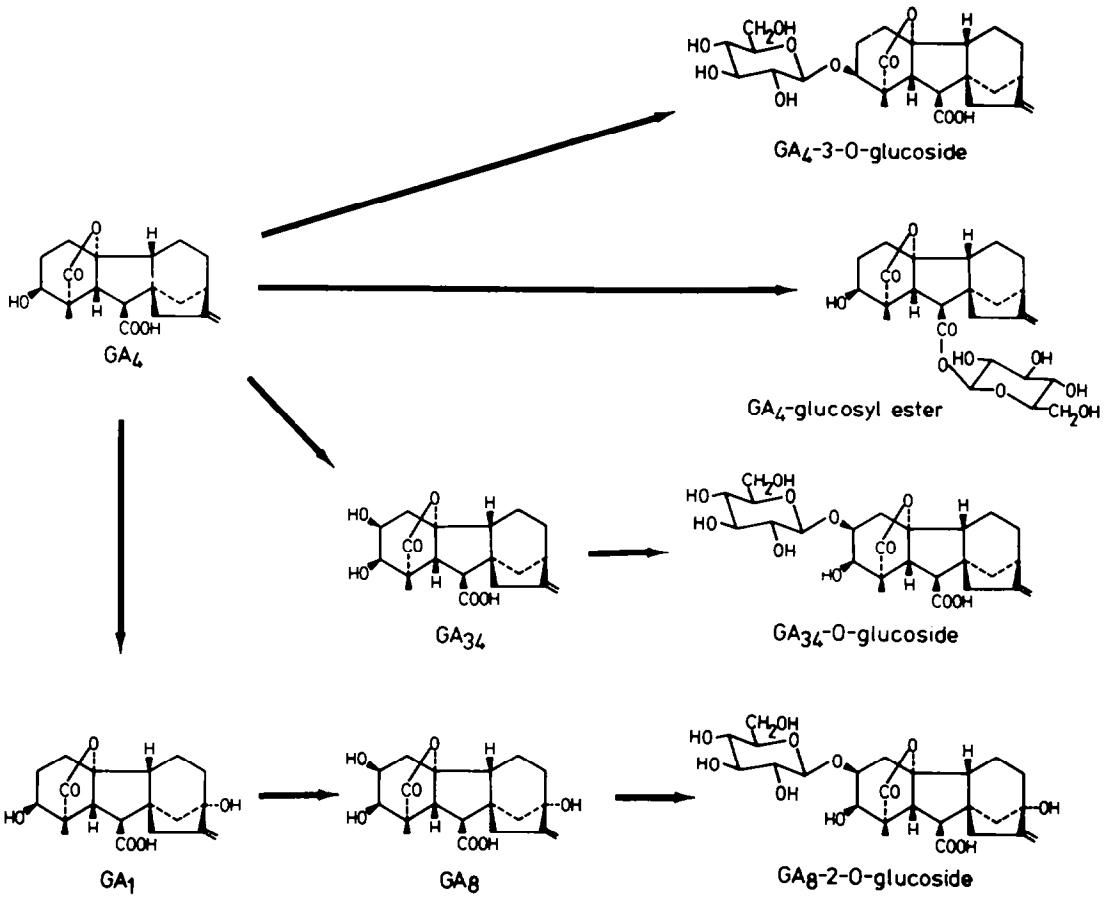


Fig. 2. Probable metabolic relationship of metabolites of [^{3}H]GA_4 fed to epicotyls of *P. coccineus* seedlings.

glycosidic linkage. It should be stressed, however, that these procedures are not suitable for the analysis of unlabelled conjugates. Reliable, routine quantitative analysis of endogenous GA conjugates is likely to depend on the development of either immunoassays or improved derivatives for GC/MS.

EXPERIMENTAL

Plant material. Seeds of *Phaseolus coccineus* L. cv. Prizewinner were soaked in running tap water for 24 hr, then stripped of their testas and germinated on H_2O-satd tissue paper in a closed chamber in a controlled environment growth room (27° constant temp., 12 hr photoperiod, light supplied by 'warm white' and 'daylight' fluorescent tubes, radiation flux *ca* 50 W/m^2 at plant height). After 5 days, an extensive root system had developed and the epicotyls were 5–12 cm long.

Substrate and feeding to plants. [1,2-^{3}H]GA_4 (3 × 10^{10} Bq/mmol) was purified by reversed-phase HPLC before use. This substrate was dissolved in MeOH and injected into epicotyls of 57 5-day-old seedlings (1 μl, 1.78 × 10^4 Bq, 590 pmol per plant; total radioactivity 1.01 × 10^6 Bq). The plants were then incubated for 4 hr in the growth room under the conditions described above, before extraction.

Extraction. Roots and cotyledons were removed, leaving the shoots (57.0 g fr. wt of epicotyls plus unexpanded primary leaves) which were frozen in liquid N_2 and ground to a powder. The powder was extracted × 4 with cold (0°) MeOH (2 × 250 ml, 2 × 100 ml) and the extracts combined. After rotary evaporation at 40°, the resultant aq. residue (0.78 × 10^6 Bq) was adjusted to pH 7.5 with triethylammonium acetate soln, and extracted × 5 with 1/2 vol. of toluene. The aq. phase was adjusted to pH 3 with HOAc, extracted × 4 with an equal vol of EtOAc then × 4 with an equal vol. of H_2O-satd butan-2-ol. After evaporation of the organic solvents, radioactivity was found to be distributed as follows: toluene extract, 4.0 × 10^4 Bq (discarded); EtOAc, 61.0 × 10^4 Bq; BuOH, 6.5 × 10^4 Bq; aq. residue, 0.3 × 10^4 Bq (discarded), representing a recovery of 71% of the total radioactivity fed to the plants.

Gel permeation chromatography (GPC). The EtOAc and BuOH fractions were dissolved in THF–MeOH (1:1) and subjected to GPC on a Biobeads SX-4 column (2 m × 25 mm) eluted with THF at a flow rate of 2 ml/min [19]. Six-ml fractions were collected from elution vol. 250–500 ml.

HPLC. Reversed-phase HPLC was used for analytical and preparative separations at several stages during the purifications. Solvent was delivered at 1 ml/min by a Spectra Physics SP8700 liquid chromatograph. Samples were introduced off-column via a Rheodyne 7125 injection valve. All separations were carried out on a 250 mm × 5 mm column packed with 5 μm ODS Hypersil eluted with mixtures of MeOH and 10 mM aq. HOAc. The following solvent programs were used: (1) 0–20 min, 20–100% MeOH; 20–25 min, 100%. (2) Isocratic 20%. (3) 30%. (4) 40%. (5) 60%. (6) 70%. (7) 75%. For analytical separations, the eluate was mixed with scintillant and passed to a Coruflow scintillation counter (ICN Tracerlab, Mechelen, Belgium) fitted with a 500 μl

spiral glass flow cell. The scintillant consisted of 10 g/l 2,5-diphenyloxazole in Triton X-100–xylene–MeOH (11:22:5). A 3:1 scintillant–eluate ratio was compatible with all mobile phase conditions and gave *ca* 15% counting efficiency for ^3H. Unlabelled free GAs and GA conjugate standards were detected with an LC871 UV monitor (Pye Unicam) set at 206 nm. For preparative separations, 0.5 min fractions were collected directly from the column or after passing through a splitter-mixer (Reeve Analytical) in which case 1.5–10% of the sample was directed to the radioactivity monitor as above and the remainder collected.

Ion exchange chromatography. DEAE Sephadex A-25 was converted to the acetate form by equilibration with 1 M NaOAc, then washed exhaustively with MeOH before being packed into a 30 mm × 10 mm column. Samples were dissolved in MeOH (< 50 μl), loaded onto the column and eluted with the following solvents: 3 ml MeOH, 9 ml HOAc–MeOH (3:200), 12 ml HOAc–MeOH (3:47), 12 ml HOAc–MeOH (3:2). Three-ml fractions were collected. Fractions 1 and 2 contained glucosyl esters, and glucosyl ethers were found in various fractions from 4 to 9.

Cellulase hydrolysis. Cellulase soln (from *Aspergillus niger*, 10 mg/ml) was dialysed against Na citrate buffer (0.1 M, pH 4.5) for 20 hr at 4°. Aliquots of 200 μl were added to aq. samples and incubated for 24 hr at 37°.

Derivatization. (a) Transesterification. Aliquots of conjugate fractions were dissolved in 5 μl MeOH to which was added 100 μl methanolic NaOMe soln (0.5 M). After 2 hr at room temp., the mixture was acidified with 30 μl HOAc. (b) Methoxycoumaryl esters. Dry samples were dissolved in 10 μl Me_2CO to which was added a crystal of K_2CO_3, bromomethylmethoxycoumarin (10 mM, 100 μl in Me_2CO) and 18-Crown-6 catalyst (10 mM, 10 μl in Me_2CO). After incubation at 60° for 2 hr in sealed tubes, the mixtures were acidified and extracted × 4 with EtOAc [20]. (c) Methylation. Neutralized samples were methylated by addition of suitable quantities of CH_2N_2 in Et_2O.

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