

Preparation of [^{14}C]-Labelled 1,2,3,4,6-Penta-O-Galloyl- β -D-Glucose and Related Gallotannins

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[U- ^{14}C]-Labelled 1,2,3,4,6-penta-O-galloyl- β -D-glucose was prepared by photoassimilation of $^{14}\text{CO}_2$ with leaves from staghorn sumac (*Rhus typhina*) in the presence of the herbicide glyphosate. Extracts of the plant material were partitioned against ethyl acetate and chromatographed on Sephadex LH-20, yielding a series of crude tri- to decagalloylglucoses. The pentagalloylglucose fraction among these was further purified by HPLC to >99% purity and a specific radioactivity of 130 kBq (3.5 μCi) per μmol . The ratio of the radioactivities in the glucose and galloyl moieties, respectively, suggested a uniform labelling pattern of the product.

Introduction

1,2,3,4,6-Pentagalloylglucose is regarded as the immediate and common precursor of gallotannins and ellagitannins, the two classes of hydrolyzable tannins. While the principle of gallotannin biosynthesis (i.e. substitution of the parent compound with additional depsidically bound galloyl residues provided by β -glucogallin as acyl donor) has been discovered several years ago (Hofmann and Gross, 1990), virtually nothing is known about the mechanisms involved in the oxidative linkage of spatially adjacent galloyl groups of pentagalloylglucose to yield the hexahydroxydiphenoyl residues that characterize ellagitannins. In recent enzyme studies directed to this challenging question a strong requirement for highly sensitive analytical procedures was encountered (cf. Gross, 1994), and it became evident that availability of the substrate 1,2,3,4,6-pentagalloylglucose with radioactively labelled galloyl moieties should offer a powerful and perhaps indispensable tool. The chemical synthesis

of such a compound, e.g. by an adaptation of the four-step procedure published by Gross (1983), did not appear very promising considering the limited availability of labelled gallic acid and the enormous problems related to scaling down a method that had been designed for the preparation of gram quantities of unlabelled material. As an attractive alternative, we adapted a method for the synthesis of labelled shikimic acid by photoassimilation of $^{14}\text{CO}_2$ in the presence of glyphosate (Krüper *et al.*, 1990); this herbicide blocks a key enzyme of the shikimate pathway, 5-enolpyruvylshikimate 3-phosphate synthase, and thus causes the accumulation of shikimic acid in plant tissues. It has been reported (Amrhein *et al.*, 1984; Lydon and Duke, 1988; Becerril *et al.*, 1989) that also the levels of gallic acid increased considerably after application of glyphosate to various plants (direct aromatization of shikimic or dehydroshikimic acid is supposed to represent a significant, if not the major route to gallic acid; reviewed, e.g., by Gross, 1992a, 1992b), and these findings prompted us to include this agent in our experiments to enforce an increased flow of ^{14}C into galloylated products. As a prerequisite for future studies on the biosynthesis of ellagitannins and related compounds, we report here on the synthesis and purification of [U- ^{14}C]1,2,3,4,6-pentagalloylglucose that was prepared in satisfying yield and specific radioactivity by photoassimilation of $^{14}\text{CO}_2$ in leaves of *Rhus typhina*.

Abbreviations: GG, galloylglucose; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography.

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Materials and Methods

Chemicals

Reference samples of 1,2,3,4,6-pentagalloylglucose and related galloylglucoses were from the laboratory collection, obtained by chemical synthesis and isolation from natural sources (cf. Hofmann and Gross, 1990) or as generous gifts of Professors G. Nonaka (Fukuoka, Japan) and E. Haslam (Sheffield, UK). Glyphosate was kindly provided by Professor N. Amrhein (Zürich). Tannase was a gift of Dr. Kanaoka (Sankyo Co., Tokyo).

Plant material

Prior to sowing, seeds of staghorn sumac (*Rhus typhina* L.) were soaked in conc. H_2SO_4 to break dormancy (Rolston, 1978); the developing seedlings were raised in the greenhouse under natural light conditions (day/night temperature 22/19 °C). Shoots of 1-year-old plants were used for the experiments.

Analytical methods

Normal-phase HPLC was carried out on LiChrosorb Si-60 (Merck; 180 × 3 mm i.d. CGC-glass cartridges, particle size 5 µm) with the solvent *iso*-hexane-methanol-tetrahydrofurane-formic acid (56:33:11:1; by vol.) plus 400 mg oxalic acid per litre (modified after Nishizawa *et al.*, 1980). Reversed-phase HPLC was performed on LiChrospher RP-18 (Merck; 250 × 4 mm i.d. steel cartridges, particle size 5 µm) with a linear gradient of 10–50% acetonitrile in 0.05% aq. H_3PO_4 within 0–20 min. Flow rates were generally 1 ml/min; quantification was by UV photometry (280 nm) and a computing integrator (Merck-Hitachi D-2500) under reference to external standards. TLC was done on cellulose-coated plastic sheets (CEL-400; Macherey–Nagel, Düren) with 6% aq. acetic acid as solvent. Labelled spots of gallic acid (R_f 0.33) and glucose (R_f 0.78) were located by a thin-layer scanner (Berthold, Wildbad), cut out and directly transferred into vials for liquid-scintillation counting.

Photoassimilation

Photoassimilation was carried out in a cylindrical glass reaction vessel (vol. 10 l) covered with a gas-tight lid bearing four ground joints. Two of

these were equipped with a thermometer and a Geiger-Müller counting tube, respectively, to monitor temperature and radioactivity of the gas-phase. For the generation of $^{14}\text{CO}_2$, the third joint was closed with a serum cap to allow injection of 0.5 ml 50% aq. H_3PO_4 with a syringe and a long hypodermic needle into a suitably placed sample of solid [^{14}C]BaCO₃ (Amersham; 185 MBq, 5 mCi). The fourth joint was equipped with a stopcock and served to remove residual $^{14}\text{CO}_2$ at the end of the incubation period by suction of the gas-phase through two gas traps containing 250 ml 50% KOH. The incubator was illuminated by four ring-shaped 40 W fluorescent lamps, and a temperature of 26 °C was maintained by an external thermostated water-circulation system. For the experiment, 10 shoots of young *R. typhina* plants (total fresh weight 80 g) were placed in a beaker with 250 ml potassium phosphate buffer (10 mM, pH 6.0, plus 1 mM glyphosate), transferred to the incubator and allowed to photoassimilate $^{14}\text{CO}_2$ for one week until cessation of CO_2 -consumption was reached.

Extraction and purification of [^{14}C]1,2,3,4,6-pentagalloylglucose

The radioactive leaf material was cut into smaller pieces, destroyed in boiling methanol (2 l, 5 min) and exhaustively Soxhlet extracted with acetone (1 l), followed by 70% aq. acetone (0.5 l). The solid residue obtained after rotary evaporation of the combined methanol/acetone extracts was redissolved in water and partitioned in several portions against ethyl acetate (total vol. 3 l) for 24 h in a Kutscher-Steudel perforator. Evaporation of the organic phase yielded 1.7 g crude tannin from which 1 g was redissolved in ethanol (12 ml) and purified in two portions by chromatography on Sephadex LH-20 (Pharmacia; column size 450 × 30 mm i.d.) as described by Nishizawa *et al.* (1980). Rechromatography of the pentagalloylglucose fractions (100 mg) afforded 60 mg of partially purified 1,2,3,4,6-pentagalloylglucose which was dried, redissolved in ethanol and subjected in small aliquots to semi-preparative reversed-phase HPLC (Merck LiChrosorb RP-18, particle size 5 µm; column 250 × 7 mm i.d.; linear gradient of 10–50% acetonitrile in 0.05% aq. H_3PO_4 within 20 min; flow rate 2 ml/min). The eluates were gently

concentrated by short (2 h) lyophilization in a spin-freeze dryer to remove the organic solvent. The residual aqueous phase was deacidified by adsorption on LiChrosorb RP-18 (Merck; particle size 40–63 μm , column 60 \times 15 mm i.d.; equilibrated in H_2O); after washing with 10 ml water, elution with 5 ml acetonitrile afforded 20 mg (23.8 μmol) 1,2,3,4,6-pentagalloylglucose with a specific radioactivity of 130 kBq (3.5 μCi) per μmol in >99% purity.

Results and Discussion

Leaves of staghorn sumac (*Rhus typhina*) were chosen for the photoassimilation of $^{14}\text{CO}_2$ as this plant is known for the effective *in vivo* synthesis of the desired product, 1,2,3,4,6-pentagalloylglucose. We were aware that the significant endogenous levels of gallotannins in the plant material would prevent extreme specific radioactivities of the product; it was assumed, however, that high levels of administered labelled precursor together with prolonged incubation should be sufficient to achieve at least satisfying results.

Under this premise, $^{14}\text{CO}_2$ was fed to the leaves as described in the experimental section. The extracted crude material was subjected to column chromatography on Sephadex LH-20. Dried aliquots of the eluates were assayed by normal-phase HPLC according to Nishizawa *et al.* (1980), a method that allows rapid analyses but has the dis-

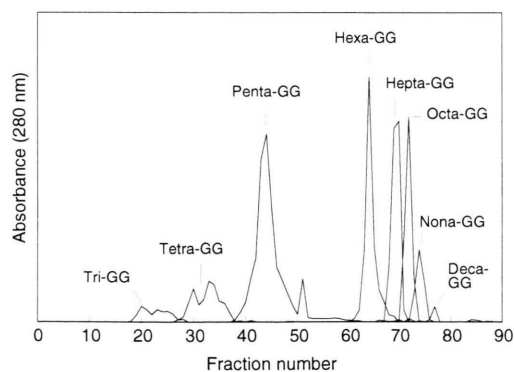


Fig. 1. Column chromatography of crude [^{14}C]labelled gallotannins from sumac leaves on Sephadex LH-20 (Nishizawa *et al.*, 1980). The depicted substitution degrees and concentrations of eluted galloylglucoses were determined by normal-phase HPLC on Si-60 silica gel. Details are given in the experimental section. GG, galloylglucose.

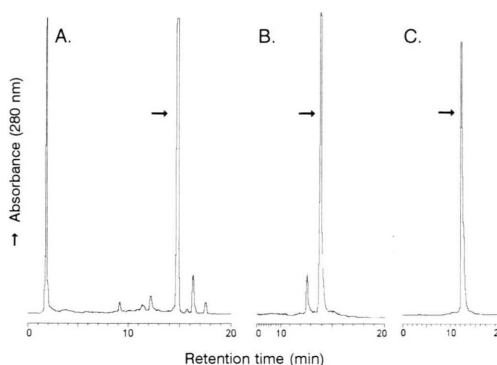


Fig. 2. Reversed-phase HPLC-analysis of [^{14}C]1,2,3,4,6-pentagalloylglucose at different purification steps. **A:** Product after first chromatography on LH-20; **B:** after rechromatography on LH-20; **C:** purified product after semi-preparative HPLC on RP-18. Pentagalloylglucose is marked by arrows. For details see Materials and Methods.

advantage of separating compounds only according to their galloylation degrees. As shown in Fig. 1, a series of increasingly substituted gallotannins was isolated, yielding 50 mg tri-GG, 70 mg tetra-GG, 100 mg penta-GG, 120 mg hexa-GG, 120 mg hepta-GG, 40 mg octa-GG, 15 mg nona-GG and 10 mg deca-GG. The pentagalloylglucose fraction among these was rechromatographed under identical conditions to yield a 100% pure product (30 mg) according to the criteria of the above assay, while closer analysis by reversed-phase HPLC revealed a concentration of 86.5% for 1,2,3,4,6-pentagalloylglucose. Final purification was achieved by semi-preparative HPLC on RP-18 silica gel affording pure 1,2,3,4,6-pentagalloylglucose which was depleted of organic solvent and acid before evaporation to dryness. The entire purification sequence, the progress of which is shown in Fig. 2, yielded 20 mg of >99% pure 1,2,3,4,6-pentagalloylglucose with a specific radioactivity of 130 kBq (3.5 μCi) per μmol , a value which is sufficiently high for most biosynthetic investigations with cell-free systems.

The UV spectrum of the isolated product (in ethanol) was identical with that of a standard sample. Degradation with tannase (cf. Hofmann and Gross, 1990) and subsequent TLC of the reaction products afforded gallic acid and glucose as sole labelled components in a radioactivity ratio of 7.5 : 1. Uniform distribution of ^{14}C , as expected from the employed synthetic procedure, would require

a ratio of 5.8 : 1; however, this moderate discrepancy is not necessarily contradictory to the assumption that in fact [^{14}C]pentagalloylglucose had been formed in the assimilation reaction.

In summary, a comparatively simple, effective and economic method for the synthesis of [^{14}C]labelled 1,2,3,4,6-pentagalloylglucose has been developed which is also applicable without major alterations for the preparation of other gallotannins. It thus represents an extension of previous reports

on the enzymatic synthesis of [^{14}C]glucosyl or [^{14}C]galloyl-labelled β -glucogallin and of 1,6-digalloyl-[^{14}C]glucose (Gross, 1983; Denzel *et al.*, 1988; Gross and Denzel, 1991).

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