

## Dihydrokaempferol as Precursor for Catechins in the Tea Plant

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The incorporation of [G-<sup>3</sup>H]-dihydrokaempferol into catechins of young tea shoots (*Camellia sinensis* L.) was compared with that of L-[U-<sup>14</sup>C]-phenylalanine and [U-<sup>14</sup>C]-shikimic acid. The percent incorporation of all three precursors into (—)epicatechin was about equal, whereas the incorporation of shikimic acid into the galloylated catechins was somewhat higher than that of phenylalanine and dihydrokaempferol. From the results it can be concluded that dihydrokaempferol is a precursor for catechins in the tea plant.

It had been shown previously that [ $\beta$ -<sup>14</sup>C]-4, 2', 4', 6'-tetrahydroxychalcone-2'-glucoside<sup>1</sup> and the corresponding flavanone [2-<sup>14</sup>C]-naringenin<sup>2</sup> (5, 7, 4'-trihydroxyflavanone) are incorporated into catechins in young tea leaves or shoots. With the tetrahydroxychalcone-glucoside as precursor it was proved that the incorporation into epicatechin occurred without randomization of the label<sup>1</sup>. Dihydrokaempferol and dihydroquercetin, which proved to be efficient precursors for kaempferol and quercetin<sup>3,4</sup> and for cyanidin<sup>5</sup>, have also been postulated to be the more immediate precursors for catechins<sup>6</sup>.

In the present paper we show that [<sup>3</sup>H]-dihydrokaempferol is incorporated into catechins of young tea shoots with about the same efficiency as [<sup>14</sup>C]-shikimic acid and L-[<sup>14</sup>C]-phenylalanine.

### Materials and Methods

#### Plant material

Fresh young tea shoots of the Georgian tea-plant varieties (*Camellia sinensis* L.) were collected in the middle of July at 10 o'clock in the morning near Sukhumi (Caucasus)).

#### Radioactive precursors

L-[U-<sup>14</sup>C]-phenylalanine (118 mCi/mm was a commercial sample (Praha, Institute for research produc-

tion and uses of radioisotopes). Chromatographically pure [U-<sup>14</sup>C]-shikimic acid (0,035 mCi/mm was obtained by microbiological synthesis (*Escherichia coli* 83-24) from D-[U-<sup>14</sup>C]-glucose<sup>7</sup>. [G-<sup>3</sup>H]-Dihydrokaempferol (6 mCi/mm) was obtained by a modified Wilzbach technique<sup>8</sup>.

#### Precursor solutions

L-[U-<sup>14</sup>C]-phenylalanine (0,5 mCi; 0,7 mg) was mixed with unlabelled L-phenylalanine (12,5 mg). The mixture was dissolved in 3 ml of water, and 5 ml of a 0,01% chloramphenicol solution was added. [U-<sup>14</sup>C]-Shikimic acid (0,009 mCi; 44,0 mg) was dissolved in 3 ml of water. This solution was adjusted to pH 6,2 with diluted NH<sub>4</sub>OH, and 0,01% chloramphenicol solution was added up to a total volume of 8 ml. [G-<sup>3</sup>H]-Dihydrokaempferol (0,073 mCi; 3,5 mg) was mixed with 2 ml of water to which two drops of Tween-80, ascorbic acid (1,0 mg) and 0,01% chloramphenicol solution were added to make a volume of 6 ml.

#### Administration of precursors

The precursor solution was administered through the cut stems of the shoots in small plexiglas cells. 10-12 shoots were placed in 3-4 ml of solution in daylight. After the solution had been taken up (1,5 to 2 hours) 4 ml of water was added to each cell and the shoots were then incubated for another 6 hours. The shoots were then washed with water and the tip of the stems was removed. The shoots were subsequently treated with steam (105 °C) for 2,5 min and then dried at 75–80 °C. In the experiment with dihydrokaempferol about 20% of the stem tips turned brown. These parts were removed before the steam treatment.

#### Isolation of flavonoids

The dried, coarsely powdered plant material (about

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4-5 g) was extracted for 20 hours with benzene-chloroform (1:1) in a Soxhlet apparatus. After removal of the solvent *in vacuo* the plant material was extracted twice with hot oxygen-free water (each time 20 min in darkness at 95 °C). The combined water extracts were filtered through a small polyamide column (Woelm, Eschwege; 20 g; 4,4, x 4 cm) and the column was washed with distilled water until negligible radioactivity remained in the filtrate. Flavonoids were then eluted with 96% ethanol. The first fractions of the eluate usually contained kaempferol-glycosides and part of the quercetin-glycosides, followed by galloylated catechins, simple catechins and myricetin glycosides.

The ethanolic solutions were evaporated *in vacuo* and the residue was dissolved in a small amount of water. Water solutions were freeze-dried. All flavonoid samples were kept in darkness in sealed tubes.

#### Separation of catechins and flavonol-glycosides

The flavonoid fractions containing catechins were repeatedly extracted with 5-8 ml portions of water-saturated ether until the ether extract gave only a weak colour with vanillin-HCl (1% in conc. HCl). The combined ether solutions containing catechins were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and the ether was removed *in vacuo*.

The ether-insoluble fraction containing the flavonol-glycosides and small amounts of condensed catechins was dissolved in 10 ml of water.

#### Chromatographic separation of catechins and flavonols

For descending paper chromatography on Whatman 3 MM, prewashed with 0.01 M EDTA, the following solvent systems were used: 1. Butanol-acetic acid-water (40:12:28, by vol.) and 2. 5% acetic acid. Flavonol aglyca do not move in solvent system 2 and can therefore be separated from catechins  $R_F = 0.25-0.80$ . In solvent system 1 the following  $R_F$ -values are found: (—)Epigallocatechin, 0.45; (+)-gallocatechin, 0.52; (—)-epicatechin, 0.62; (—)-epigallocatechingallate, 0.63; (—)-epicatechingallate, 0.80; quercetin, 0.70; and myricetin, 0.56.

Sharper spots are obtained by thin-layer chromatography on cellulose (Avicel, Merck AG, Darmstadt). The relative mobility of the compounds are the same.

The galloylated catechins give a pale-blue fluorescence in UV-light after treatment with ammonia vapor. Simple catechins were detected with 5% vanillin in conc. HCl.

#### Radioactive measurements

Samples were dissolved in a toluene cocktail (5 g PPO, 1 l toluene) and counted in a scintillation spectrometer.

### Results and Discussion

Solutions of L-[U-<sup>14</sup>C]-phenylalanine, [U-<sup>14</sup>C]-shikimic acid and [G-<sup>3</sup>H]-dihydrokaempferol were fed through the cut stems of young tea shoots. After 8 hours of incubation in daylight catechins were isolated and purified to constant specific activity as described under "Methods". The percent incorporation of these precursors into free and galloylated catechins are recorded in the Table. The determination of dilution values was not meaningful in this case<sup>9</sup> since the molar amounts of the applied precursors are quite different (dihydrokaempferol: phenylalanine: shikimic acid, 1:6.5:20.6) owing to the low specific activity of shikimic acid.

The incorporation values show that dihydrokaempferol is as good a precursor for catechins as phenylalanine. The incorporation of shikimic acid was somewhat higher in the case of galloylated catechins. This may be due to the additional incorporation of this acid into the gallic acid residue. It has been shown that in higher plants gallic acid is derived mainly or exclusively from dehydroshikimic acid<sup>10,11</sup>.

It can be concluded from these results that dihydrokaempferol is an intermediate in the biosynthesis of catechins from tetrahydroxychalcone in tea leaves (Fig. 1).

The process by which the carbonyl-oxygen at C-4 is removed is unknown. Further insight into this

Table. Incorporation of various precursors into catechins of tea leaves.

Precursor Compound	[U- <sup>14</sup> C]-Shikimic acid	l-[U- <sup>14</sup> C]-Phenylalanine	[G- <sup>3</sup> H]-Dihydrokaempferol
	Incorporation [%]	Incorporation [%]	Incorporation [%]
(—)epicatechin	0.14	0.10	0.11
(—)epigallocatechin	0.80	0.29	—
(—)epicatechingallate	1.95	—	0.48
(—)epigallocatechingallate	3.70	1.30	1.13

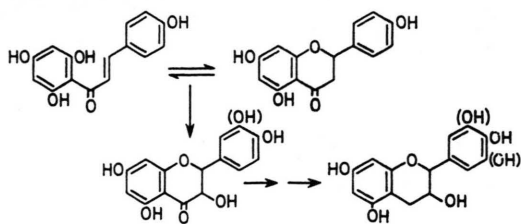


Fig. 1. Postulated biosynthetic pathway from chalcone to catechins via dihydroflavonol.

pathway can probably be gained only with a cell-free system.

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