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4′-*O*-β-D-GLUCOSYL-*CIS*-*p*-COUMARIC ACID—A NATURAL CONSTITUENT OF *SPHAGNUM FALLAX* CULTIVATED IN BIOREACTORS

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Key Word Index—*Sphagnum fallax*; Sphagnaceae; bryophyta; *trans*-sphagnum acid; 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid; UDPG:*cis*-*p*-coumaric acid glucosyltransferase.

Abstract—Exogenously applied *p*-coumaric acid (0.1 mM) was rapidly taken up by *Sphagnum fallax* batch cultures, precultivated axenically in bioreactors, and metabolized to *trans*-sphagnum acid and a *p*-coumaric acid conjugate. The structure of the isolated conjugate was elucidated by NMR and IR spectroscopy and was shown to be 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid. Small amounts (80–160 nmol g^{-1} dry wt) of this component are also always detectable in the homogenate of untreated *S. fallax* plantlets. Acid hydrolysis (N HCl) of the isolated glucoside or treatment with a β-glucosidase from almonds yielded mainly *cis*-*p*-coumaric acid. Irradiation of the *cis*-glucoside with UV light ($\lambda = 254$ nm) resulted in the formation of 4′-*O*-β-D-glucosyl-*trans*-*p*-coumaric acid. The biosynthesis and accumulation of 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid is dependent on the availability of *cis*-*p*-coumaric acid in the external medium; exogenously in the dark applied *trans*-*p*-coumaric acid is not glucosylated, whereas *trans*-sphagnum acid is synthesized from *trans*-*p*-coumaric acid and accumulated in the light as in the dark. A cytosolic uridine diphosphate glucose (UDPG):*p*-coumarate glucosyltransferase, which exhibits an unusual substrate specificity for *cis*-*p*-coumaric acid, was isolated from *Sphagnum fallax*. The biosynthesis of phenylpropanoids in *Sphagnum* known up to now is discussed based on the present results.

INTRODUCTION

All *Sphagnum* species analysed are characterized by a high amount of free endogenous *trans*-sphagnum acid (-*p*-hydroxy-β-[carboxymethyl]-cinnamic acid), a cinnamic acid derivative unique to peat mosses [1–4]. Hydroxybutenolide [(−)2,5-dihydro-5-hydroxy-4-(4′-hydroxphenyl)-furan-2-one], a butenolide also exclusively described for *Sphagna* [5], *p*-hydroxyacetophenone, *p*-hydroxybenzoic acid, *p*-coumaric acid and *trans*-cinnamic acid are the phenolics mainly localized in the non-lignified cell walls of *Sphagnum* from natural sites. Recent studies revealed that *trans*-sphagnum acid can be detected predominantly in the buffer soluble fraction and to a great extent also in the external medium, especially in plantlets cultivated in continuous feed bioreactors [6]. The application of the inhibitor glyphosate as well as tracer experiments with [^{3}H]-L-phenylalanine and sodium [^{14}C]acetate gave evidence that the C_6–C_3 skeleton of *trans*-sphagnum acid is synthesized via the shikimate pathway; the carboxymethyl side chain is derived from acetate [4, 7]. Exogenously supplied precursors like L-phenylalanine, *trans*-cinnamic acid and *p*-coumaric acid resulted in a strong enhancement of *trans*-sphagnum acid excretion and endogenous accumulation [8]. The postulated *trans*-sphagnum acid synthase has not yet been isolated, although a cell free system synthesizing *trans*-sphagnum acid from *p*-coumaric acid and acetate has been described previously [7].

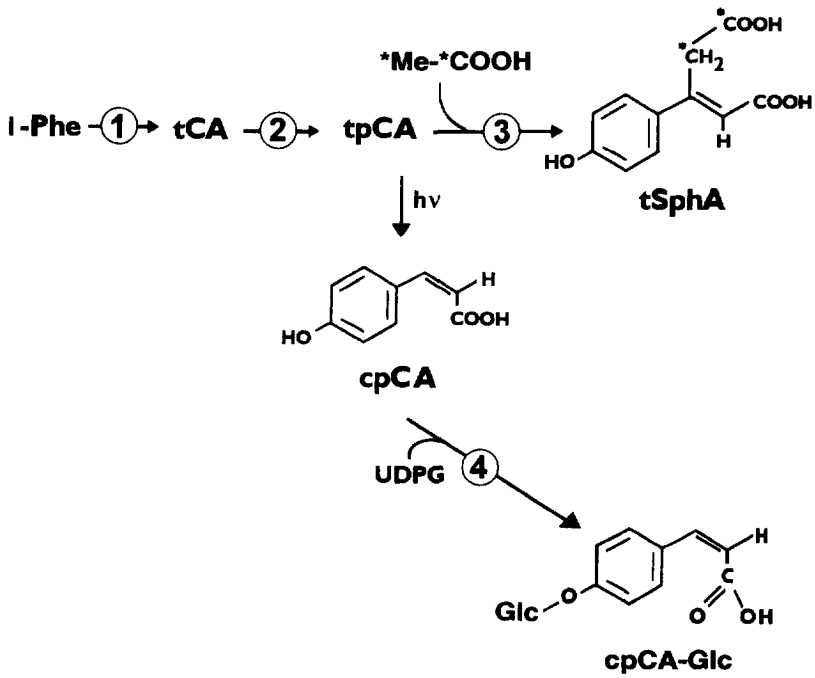
A pathway for *trans*-sphagnum acid biosynthesis, involving the enzymic reactions of L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) and cinnamic acid 4-hydroxylase (CA4H, EC 1.14.13.11), well known enzymes of the phenylpropanoid metabolism in higher plants, was proposed. However, application of *trans*-*p*-coumaric acid and *trans*-cinnamic acid to batch cultures of *Sphagnum fallax* did not only stimulate the biosynthesis of *trans*-sphagnum acid, but resulted also in the formation of large amounts of a *p*-coumaric acid conjugate, which was accumulated endogenously and excreted as well [8]. The aim of the present paper is to elucidate the structure of this component and to study the enzymology of its biosynthesis. In this con-

RESULTS AND DISCUSSION

Isolation of 4′-O-β-D-*glucosyl*-cis-p-*coumaric acid*

Exogenously supplied *trans-p*-coumaric acid (0.1 mM) was rapidly taken up by *S. fallax* batch cultures; after 48 hr up to 79% was removed from the external medium. No free endogenous *p*-coumaric acid was accumulated, 13% of the *p*-coumaric acid taken up was metabolized to *trans*-sphagnum acid, and 77% to an unknown *p*-coumaric acid conjugate [8]. HPLC analysis of the homogenate and the culture medium after 48 hr incubation of *S. fallax* batch cultures in nutrient solution supplied with 0.1 mM *p*-coumaric acid demonstrated the accumulation of the main products *trans*-sphagnum acid and *p*-coumaric acid conjugate. In the homogenate and in the culture medium of *S. fallax* batch cultures incubated as controls without *p*-coumaric acid, the conjugate was also detectable, but to a much smaller extent. The *p*-coumaric acid conjugate was predominantly accumulated endogenously and was isolated from buffer homogenates of incubated *S. fallax* plantlets by semi-preparative reversal phase (RP)-HPLC. The fractions containing the conjugate were collected, stored (−18°) and concentrated in the dark to avoid any light dependent isomerization during the purification procedure.

By means of NMR and IR spectroscopy the structure was shown to be 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid (**1**) (Fig. 1). The ^{1}H NMR spectrum shows the characteristic doublets for two protons, respectively, of a *p*-substituted phenyl moiety at 7.10 and 7.47 ppm. Two doublets at 6.01 and 6.71 ppm for one proton in each case prove the presence of an additional 1,2-disubstituted double bond; the *cis*-configuration is verified by the $^{3}J_{HH}$-coupling constant of 12 Hz. Two doublets at 5.16 and 3.94 ppm for one proton, respectively, and three multiplets at 3.76, 3.63 and 3.52 ppm for altogether five protons represent a β-glucosyl moiety. These results were verified and complemented by the ^{13}C NMR spectrum, which shows two signals (*d*) for two carbon atoms at 131.0 and 117.1 ppm and two additional (*s*) at 157.2 and 131.4 ppm of a 1,4-disubstituted phenoxy moiety. Three signals at 153.3 (*d*), 124.1 (*d*) and 175.6 (*s*) ppm prove the above postulated olefinic C=C group as an α,β-unsaturated carbon acid. The characteristic signals of a glucosyl moiety are at 100.8 (*d*), 77.0 (*d*), 76.4 (*d*), 73.8 (*d*), 70.3 (*d*) and 61.4 ppm (*t*), which are in good accordance with those of the glucosyl moiety of phenyl-4-β-D-glucoside [9]; slight divergences are probably due to the low concentration of the measured sample. The presence of a glucosyl ester can be ruled out, since the resonance of the carboxylic carbon atom (175.6 ppm) differs from that of an ester (*cis-p*-coumaric acid ethyl ester: 167.4 ppm). This view is supported by the heteronuclear multiple bond correlation (HMBC) spectrum [10] of the ^{1}H and ^{13}C NMR spectra of the isolated compound, in which on a waiting time Δ3 = 63 msec, corresponding to a $^{3}J_{HC}$-coupling constant of *ca* 8 Hz, a correlation signal of the anomeric 1″-H of the glucosyl moiety can only be found at the C-4′ of the phenyl moiety and not at the C-1 of the carbon acid



function. This is further confirmed by the IR spectrum, in which no band between 1700 and 2700 cm^{-1} appeared, but an intense shoulder at 1690 cm^{-1} did, probably due to a C=O valence vibration not sufficiently resolved. The unexpected highfield shift of C-3 (135.3 ppm) and the lowfield shift of C-2 (124.1 ppm) in comparison with cinnamic acid spectra (usually 145 and 116 ppm, respectively), and the signal broadening of the carboxylic C-atom C-1 and the olefinic C-2 and C-3 may be due to a specific structural feature of 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid in which the carbonyl group is probably turned out of the plane and thereby restricts the orbital overlapping of the double bond system. The chemical shifts would then be similar to that of unconjugated systems. On the other hand, the ^{13}C NMR spectrum of synthesized and irradiated *cis*/*trans*-*p*-methoxycinnamic acid displayed the typical chemical shifts of cinnamic acids with 145.6 and 116.1 ppm for the conjugated double bond and 171.5 ppm for the carboxylic carbon atom. The resonance of the carboxylic carbon-atom of *cis*-*p*-coumaric acid ethyl ester was, as expected, at 167.4 ppm.

Chemical and enzymatical characterization of the isolated glucoside

The isolated and purified glucoside was hydrolysed under various conditions and the resulting products analysed by RP-HPLC (Table 1). The main product displayed the same UV-spectrum in each case as *cis*-*p*-coumaric acid obtained from purchased and irradiated *trans*-*p*-coumaric acid. Acid hydrolysis of the conjugate, performed in the dark, yielded, more quickly and at lower temperatures, more *cis*-*p*-coumaric acid than did alkaline hydrolysis. The 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid was rapidly hydrolysed by a β-glucosidase from almonds. An esterase from pig liver showed no activity towards the glucoside.

The purified *cis*-glucoside was partially converted into the *trans*-glucoside by irradiation with UV light ($\lambda = 254$ nm), subsequent hydrolysis of the isomer mixture yielded, apart from *cis*-*p*-coumaric acid, also large amounts of *trans*-*p*-coumaric acid (Table 2). These results rule out that the isolated 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid was isomerized as a consequence of the purification procedure or by photochemical means.

In vivo *studies on the biosynthesis of* 4′-O-β-D-*glucosyl*-cis-p-*coumaric acid with exogenously supplied* p-*coumaric acid*

The incubation of *S. fallax* batch cultures with *trans*-*p*-coumaric acid as described above took place under the light conditions used for plant material cultivated in bioreactors. Under these conditions, after a 14-hr light period, 52% of *trans*-*p*-coumaric acid dissolved in nutrient solution (0.1 mM, pH 3.5) is isomerized to its *cis*-isomer. To establish whether the *cis*-*p*-coumaric acid glucoside is formed from *trans*- or *cis*-*p*-coumaric acid, we compared the effects of exogenously applied *trans*-*p*-coumaric acid with that of an isomer mixture of *cis*-/*trans*-*p*-coumaric acid on cultures incubated in the dark or the light for 14 hr. The total uptake of *p*-coumaric acid from the external medium turned out to be essentially the same in each assay (Table 3). However, the uptake of *cis*-*p*-coumaric acid, if available, was favoured to that of *trans*-*p*-coumaric acid; 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid was the main product of exogenously applied *p*-coumaric acid in those assays containing *cis*-*p*-coumaric acid. In batch cultures incubated with *trans*-*p*-coumaric acid in the dark, very low amounts (300 nmol g^{-1} dry wt), com-

Table 1. Chemical and enzymic hydrolysis of 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid from *S. fallax* buffer homogenates (all reactions were carried out in the dark)

Method of hydrolysis	Time (hr)	cpCA-Glc* (μmol ml^{-1})	*cis*-pCA† (μmol ml^{-1})	*trans*-pCA (μmol ml^{-1})
N NaOH (56°)	0	1.20	—	—
	10	1.18	0.02	0.00
	20	1.15	0.04	0.01
N NaOH (95°)	0	1.20	—	—
	1	0.89	0.28	0.03
	5	0.10	1.02	0.08
N HCl (56°)	0	1.2	—	—
	10	0.72	0.34	0.11
	20	0.07	1.02	0.14
N HCl (95°)	0	1.20	—	—
	1	0.00	1.01	0.19
β-Glucosidase	0	2.50	—	—
	0.5	1.30	1.08	0.12
	1	0.14	2.26	0.24
Esterase	0	1.80	—	—
	1	1.80	0.00	0.00

Table 2. Chemical and enzymic hydrolysis of irradiated 4′-*O*-β-D-glucosyl-*p*-coumaric acid. The isomers were separated by RP-HPLC, the UV spectra measured in the λ_{scan}/stop flow mode

		Isomeric glucosides		Hydrolysis products	
	Time (hr)	cpCA-Glc* (μmol ml^{-1})	tpCA-Glc† (μmol ml^{-1})	*cis*-pCA‡ (μmol ml^{-1})	*trans*-pCA, (μmol ml^{-1})
N NaOH (95°)	0	0.70	0.50	—	—
	2	0.37	0.27	0.31	0.25
	5	0.06	0.04	0.54	0.56
N HCl (95°)	0	0.70	0.50	—	—
	1	0.00	0.00	0.59	0.61
β-Glucosidase	0	1.50	1.00	—	—
	0.5	0.78	0.52	0.69	0.51
	1	0.08	0.06	1.15	1.21
Absorption maximum (nm)		282	292	296	310

*cpCA-Glc = 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid.
†tpCA-Glc = 4′-*O*-β-D-glucosyl-*trans*-*p*-coumaric acid.
‡pCA = *p*-coumaric acid.

parable to that of untreated continuous feed bioreactor plant material (80–160 nmol g^{-1} dry wt), were detected. In contrast, formation of *trans*-sphagnum acid was about the same in all assays. These results prove that the *cis*-*p*-coumaric acid glucoside is indeed formed from *cis*-*p*-coumaric acid whereas *trans*-sphagnum acid is synthesized from *trans*-*p*-coumaric acid. Furthermore, the isomerization of *trans*-*p*-coumaric acid is presumably photochemical and not enzyme-mediated. In Fig. 1 the biosynthesis of 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid and *trans*-sphagnum acid in *Sphagnum* is summarized according to the present results.

No *trans*-*p*-coumaric acid glucosides or esters were detected in *Sphagnum* as is described for the cinnamic acid metabolism in, e.g. *Cestrum poeppigii* [11, 12] or in suspension cultures of *Phaseolus vulgaris* [13]. Photoisomerization of cell wall bound *trans*-ferulic acid to *cis*-ferulic acid was described for coleoptiles and primary leaves of barley seedlings [14] and *Triticum aestivum* [15]. An indirect or direct photoisomerization of cell wall ferulate and diferulate carbohydrate esters was suggested to be a mechanism for transduction of light energy, leading to changes in wall structure and phototropism [16]. However, the photoisomerization described in these cases took place after the esterification of *trans*-hydroxycinnamic acids; to date, no information is available with regard to an enzymic turnover of *cis*-hydroxycinnamic acids *in vivo*.

In vitro *studies on the biosynthesis of* 4′-O-β-D-*glucosyl*-cis-p-*coumaric acid*

The rapid formation of large amounts of the *cis*-*p*-coumaric acid glucoside in *S. fallax* cultures supplied

Table 3. Recovery of *p*-coumaric acid from the external medium, endogenous accumulation and excretion of *trans*-sphagnum acid and 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid in *S. fallax* batch cultures after 14 hr incubation in nutrient solution supplied with 0.1 mM *p*-coumaric acid in the dark or light

	Recovery of pCa* from the external medium (μmol culture^{-1})		Endogenous phenolics (μmol culture^{-1})		Excreted phenolics (μmol culture^{-1})	
Applied pCA-isomers (μmol culture^{-1}, light conditions)	*trans*-pCA	*cis*-pCA	*cis*-pCA-Glc†	*trans*-SphA‡	*cis*-pCA-Glc	*trans*-SphA
48 μmol *trans*-pCA (14 hr, dark)	30.0	0.0	0.3	2.5	0.1	0.2
23 μmol *trans*-pCA, 25 μmol *cis*-pCA (14 hr, dark)	19.0	11.0	5.2	1.9	0.5	0.2
48 μmol *trans*-pCA (14 hr, light)	18.0	11.0	5.9	2.5	0.5	0.4
23 μmol *trans*-pCA, 25 μmol *cis*-pCA (14 hr, light)	18.0	11.0	6.0	1.9	0.6	0.4

with exogenous *p*-coumaric acid suggested the presence of a glucosyltransferase with activity towards *cis*-*p*-coumaric acid. An *in vitro* enzyme assay was best carried out with a protein fraction which had been precipitated with ammonium sulphate (40–65%). The desalted crude enzyme was incubated in the dark with UDPG (final concentration: 7.5 mM) and either *trans*-*p*-coumaric acid (final concentration: 6 mM) or an irradiated solution containing *cis*- and *trans*-*p*-coumaric acid (final concentrations: 2 and 4 mM, respectively). Apart from *p*-coumaric acid, *cis*- and *trans*-isomer mixtures of cinnamic acid, *p*-coumaric acid ethyl ester and *p*-methoxycinnamic acid were tested as substrates. The UDPG: *p*-coumaric acid glucosyltransferase was specific for *cis*-*p*-coumaric acid; no turnover of any other cinnamic acid derivative tested was detected. The formation of 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid was linear with respect to time up to 5 hr. Frozen (−18°) desalted crude enzyme solutions showed no loss in activity for at least five weeks. Measured specific activities were in the range 8–15 pkat mg^{-1} protein.

To prove the identity of the product formed from *cis*-*p*-coumaric acid and UDPG by the enzymic reaction of the glucosyltransferase from *S. fallax*, we isolated and purified the glucoside from concentrated assay solutions by semi-preparative RP-HPLC and confirmed its structure by ^{1}H and ^{13}C NMR spectroscopy. The NMR spectra displayed essentially the same signals as the spectra of the 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid isolated from concentrated homogenates of *S. fallax* incubated with *p*-coumaric acid *in vivo*.

The isolation and purification of a UDP-glucose: coniferyl alcohol glucosyltransferase was described for the first time from cell suspension cultures of Paul's scarlet rose [17]. This glucosyltransferase seems to be ubiquitously distributed in plants and was also detected in *S. compactum* [18]. Although it was not explicitly mentioned, if the *cis*- or *trans*-cinnamyl alcohols were glucosylated, it can be assumed that the synthetic *trans*-isomers were used as substrates, no indication of photochemical reactions was given by the authors. In contrast, a glucosyltransferase was isolated from *Fagus grandifolia* [19], displaying a substrate specificity towards *cis*-coniferyl alcohol. In maturing tomatoes (*Lycopersicum esculentum* var. *cerasiforme*), an *in vitro* synthesis of esters and glucosides from free hydroxycinnamic acids (*p*-coumaric, ferulic and caffeic) and UDPG is described [20], but no evidence for a stereospecificity of the glucosyltransferase is given. From *Ipomoea batatas* [21] and *P. vulgaris* [13] a UDPG: cinnamate glucosyltransferase with a substrate specificity towards *trans*-cinnamate was isolated. Hence, this is the first report of a UDPG:*p*-coumarate glucosyltransferase, which exhibits a strong substrate specificity towards the *cis*-isomer of *p*-coumaric acid. The occurrence of the *cis*-glucoside in homogenates of *S. fallax* cultivated in continuous feed bioreactors

EXPERIMENTAL

Plant material. Sphagnum fallax (Klinggr., clone 1) was precultivated axenically in continuous feed 6 l bioreactors [22, 23]. The 4-fold concd standard nutrient soln (4-fold SNS) was applied at a flow rate of 100 ml hr^{-1}.

Application of p-*coumaric acid in batch-cultures.* A 0.1 mM soln of *trans*-*p*-coumaric acid in 4-fold SNS was prepd and the pH adjusted to 3.5. To avoid the isomerization to *cis*-*p*-coumaric acid all steps were carried out in the dark. A mixt. of *cis*- and *trans*-*p*-coumaric acid (52% *cis*- and 48% *trans*-*p*) was prepd by irradiating 0.1 mM *trans*-*p*-coumaric acid 14 hr under the same conditions used for cultivating *Sphagnum* plantlets in bioreactors (photon fluence rate: 105 μmol m^{-2} s^{-1}). The precultivated plant material was harvested from the bioreactor at the end of the light period, sepd from the external medium, and equal amounts of 4 g fr. wt (0.5 g dry wt) applied to 500 ml bioreactors filled with 480 ml 4-fold SNS, containing 48 μmol *trans*-*p*-coumaric acid or a mixt. of 25 μmol *cis*-*p*-coumaric acid and 23 μmol *trans*-*p*-coumaric acid. Control plantlets were incubated under the same conditions in 4-fold SNS without *p*-coumaric acid. After 14 hr incubation the *Sphagnum* material was sepd from the culture medium by a sieve and from the adherent medium by centrifugation (10 min, 400 *g*). The sepd moss material was used to determine the buffer soluble endogenous phenolics. The phenolics excreted into the external medium were determined in the combined adherent and culture medium.

Uptake of p-*coumaric acid and excretion of phenolics into the external medium.* The amount of *p*-coumaric acid taken up from the external medium was determined, without further prepn, in the soln by HPLC [6]. To determine the amount of excreted *trans*-sphagnum acid and 4′-*O*-β-D-glucosyl-*cis*-*p*-coumaric acid, the external medium was filtrated (membrane filter, 0.2 μm), the pH adjusted to 7.0, and the soln concd in a rotary evaporator in the dark. The residue was dissolved in 1 ml MeOH–H_2O (7:93), centrifuged (10 000 *g*, 15 min), and the phenolics in the supernatant determined by HPLC.

Soluble phenolic constituents. To determine the level of endogenous buffer soluble phenolics the plant material was homogenized in buffer as described for the prepn of the crude enzyme extract, filtrated through gauze (80 μm), denaturated (100°, 3 min) and centrifuged (10 000 *g*, 15 min). The phenolics in the supernatant were analysed by HPLC.

Preparation of crude enzyme extracts. The plant material was homogenized in buffer (50 mM MOPS–KOH, pH 7.5, 14 mM mercaptoethanol) in a ratio of 1:5 (w/v), using a cell homogenizer. The homogenate was filtered through gauze (80 μm), the filtrate was gently stirred with 1% insoluble PVP (Polyclar AT,

pptd [40–65% $(NH_4)_2SO_4$]. After centrifugation (18 000 *g*, 30 min), the pellet was resuspended in 2.5 ml buffer (50 mM MOPS–KOH, pH 7.5) and desalted on a pre-equilibrated PD-10 column. The crude enzyme was eluted with 3.5 ml buffer. Protein content was estimated by the method of ref. [24]; BSA was used as standard. All operations were performed at 4°.

Glucosyltransferase assay. Crude enzyme (350 μl) was mixed with 75 μl UDP-Glucose (50 mM in MOPS–KOH buffer, pH 7.5) and the reaction started with 75 μl *p*-coumaric acid (40 mM *trans-p*-coumaric acid or a mixt. of 40 mM *trans-/cis-p*-coumaric acid, 2:1, in MOPS–KOH buffer, pH 7.5). The isomer mixt. was prepd by irradiation of 40 mM *trans-p*-coumaric acid for 6 hr as described below. The assay was incubated at 30° and the reaction stopped by addition of 500 μl MeOH. The denaturated proteins were pptd (10 000 *g*, 15 min) and the *p*-coumaric acid glucoside in the supernatant quantitatively determined by HPLC.

Isolation of 4′-O-β-D-*glucose*-cis-p-*coumaric acid.* The plant material was incubated for 48 hr in 0.1 mM *p*-coumaric acid (20 g fr. wt, 2 l 4-fold SNS) under the same light and CO_2 conditions as used for cultivation of the bioreactor material and prepd as described above. The denatured and centrifuged supernatant was concd by lyophilization in the dark. The residue was dissolved in 7% MeOH, centrifuged (10 000 *g*, 10 min) and the supernatant sepd by interval injection/displacement RP-HPLC (5 × 200 μl per run) [25] on a semi-prep. column (C_{18}, ODSII, 5 μm). The solvent system consisted of solvent A (HCO_2H–H_2O, 1:19) and solvent B (MeOH, HPLC grade). The sepn programme was as follows: B was held at 7% at a flow rate of 2.5 ml min^{-1} for 4 min, enhanced to 15% and continuously increased to 50% at 1.8 ml min^{-1} in 28 min and to 90% in the following 3 min. The glucoside eluted at *ca* 42% B, the frs containing 4′-*O*-β-D-glucosyl-*cis-p*-coumaric acid were pooled and frozen at −18°. To avoid hydrolysis of the glucoside the acidic eluent (HCO_2H–H_2O–MeOH; 2.9:55:42,) was evapd by lyophilization. The dried glucoside was dissolved in D_2O and its structure elucidated by means of NMR and IR spectroscopy. To isolate the glucoside from the *in vitro* UDPG-glucosyltransferase assay the crude enzyme was prepd as described above and incubated with UDPG and *cis-/trans-p*-coumaric acid for 10 hr, subsequently denaturated (100°, 3 min) and centrifuged. The glucoside was purified by semi-prep. HPLC, for each run 5 × 1 ml were injected.

Synthesis of p-*coumaryl ethyl ester and* p-*methoxycinnamic acid.* The ethyl ester of *trans-p*-coumaric acid was formed from *trans-p*-coumaric acid with EtOH and catalytic amounts of *p*-toluene sulphonic acid according to the classical method of esterification [26]. Methylation of *trans-p*-coumaryl ethyl ester with CH_2N_2 in Et_2O–MeOH (4:1) and subsequent ester

methanolic solns of these compounds with UV light (254 nm, 1.5 mW cm^{-2}, 5 hr).

Conditions of hydrolysis. Acid hydrolysis of the isolated glucoside was performed in N HCl, and alkaline solvolysis in N NaOH at 56° in the dark. For enzymatic hydrolysis 100 μg purified β-glucosidase from almonds (EC 3.2.1.21, 6.9 U mg^{-1} prot., Sigma) were dissolved in 100 μl K-Pi buffer (50 mM, pH 5.0) and incubated with 900 μl 4′-*O*-β-D-glucosyl-*cis-p*-coumaric acid for 1 hr at 30°. The reaction was stopped with MeOH. A purified esterase from pig liver (10 mg/ml, Böhringer, Mannheim) was incubated with a soln of the glucoside (1:9) for 1 hr at 30°.

Isomerization of cinnamic acid derivatives by irradiation with UV light. Solns were irradiated with UV light (Sylvania UV lamp, 254 nm) in quartz glass cuvettes (30 mm distance from the lamp) with an energy fluence rate of 1.5 mW cm^{-2} (YSI-Kettering, Model 65 Radiometer).

NMR data for 4′-O-β-D-*glucosyl*-cis-p-*coumaric acid.* ^{1}H NMR (300.132 MHz, D_2O): δ 3.52 (1 H, *m*), 3.63 (3 H, *m*), 3.76 (1 H, *dd*), 3.94 (1 H, *dd*), 5.16 (1 H, *d*, O-CH-O), 6.01 (1 H, *d*, J = 12.4 Hz, 2-H), 6.7 (1 H, *d*, J = 12.4 Hz, 3-H), 7.10 (2 H, *dm*, 2′-H). ^{13}C NMR (75.47 MHz, D_2O): δ 61.4 (*t*, CH_2O), 70.3 (*d*, 1 CHO), 73.8 (*d*, 1 CHO), 76.4 (*d*, 1 CHO), 77.0 (*d*, 1 CHO), 100.8 (*d*, 1 O-CH-O), 117.1 (2 C, *d*, C-3′), 124.1 (1 C, *dbr*, C-2), 131.0 (2 C, *d*, C-2′), 131.4 (1 C, *s*, C-1′), 135.3 (1 C, *d*, C-3), 157.2 (1 C, *s*, C-4′), 175.6 (1 C, *sbr*, C-1).

NMR data of cis-p-*methoxycinnamic acid.* ^{1}H NMR (300.132 MHz, $CDCl_3$): δ 3.83 (3 H, *s*, $=CH_3$), 5.85 (1 H, *d*, J = 12 Hz, 2-H), 6.88 (2 H, *dm*, 3′-H), 6.96 (1 H, *d*, J = 12 Hz, 3-H), 7.71 (2 H, *dm*, 2′-H). ^{13}C NMR (75.47 MHz, $CDCl_3$): δ 55.3 (*q*, $=CH_3$), 113.6 (2 C, *d*, C-3′), 116.1 (1 C, *d*, C-2), 127.1 (1 C, *s*, C-1′), 132.5 (2 C, *d*, C-2′), 145.6 (1 C, *d*, C-3), 161.8 (1 C, *s*, C-4′), 171.5 (1 C, *s*, C-1).

NMR data of cis-p-*coumaric acid ethyl ester.* ^{1}H NMR (300.132 MHz, $CDCl_3$): δ 1.27 (3 H, *t*, CH_3), 4.19 (2 H, *q*, $=CH_2$), 5.81 (1 H, *d*, J = 12.6 Hz, 2-H), 6.83 (2 H, *dm*, 3′-H), 6.86 (1 H, *d*, J = 12.6 Hz, 3-H), 7.57 (2 H, *dm*, 2′-H), 7.67 (*br*, OH). ^{13}C NMR (75.47 MHz, $CDCl_3$): δ 13.9 (*q*, CH_3), 60.4 (*t*, OCH_2), 115.0 (2 C, *d*, C-3′), 116.4 (1 C, *d*, C-2), 126.7 (1 C, *s*, C-1′), 132.0 (2 C, *d*, C-2′), 143.7 (1 C, *d*, C-3), 157.3 (1 C, *s*, C-4′), 167.4 (1 C, *s*, C-1).

REFERENCES

1. Tutschek, R. and Rudolph, H. (1971) *Ber. Dtsch. Bot. Ges.* **84**, 309.
2. Tutschek, R., Rudolph, H., Wagner, P. H. and Kreher, R. (1973) *Biochem. Physiol. Pflanz.* **164**, 461.
3. Tutschek, R. (1975) *Z. Pflanzenphysiol.* **76**, 353.
4. Rudolph, H. and Samland, J. (1985) *Phytochemis-*

6. Rasmussen, S., Wolff, C. and Rudolph, H. (1995) *Phytochemistry* **38**, 35.
7. Wilschke, J., Hoppe, E. and Rudolph, H. (1990) in *Annual Proceedings Phytochemical Society of Europe*, Vol. 29. *Bryophytes their Chemistry and Chemical Taxonomy* (Zinsmeister, H. D. and Mues, R., eds), p. 253. Clarendon Press, Oxford.
8. Rasmussen, S., Peters, G. and Rudolph, H. (1995) *Physiol. Plant.* **95**, 83.
9. Bremser, W., Ernst, L., Franke, B., Gerhards, R. and Hardt, A. (1987) *Carbon-13 NMR Spectral Data*, Vol. **4**, Aufl., Spektrum 9384. Verlag Chemie VCA.
10. Bax, A. and Marion, D. (1988) *J. Magn. Res.* **78**, 180.
11. Nagels, L. and Parmentier, F. (1976) *Phytochemistry* **15**, 703.
12. Molderez, M., Nagels, L. and Parmentier, F. (1978) *Phytochemistry* **17**, 1747.
13. Edwards, R., Mavandad, M. and Dixon, R. A. (1990) *Phytochemistry* **29**, 1867.
14. Yamamoto, E. and Towers, G. H. N. (1985) *J. Plant Physiol.* **117**, 441.
15. Turner, L. B., Mueller-Harvey, I. and McAllan, A. B. (1993) *Phytochemistry* **33**, 791.
16. Towers, G. H. N. and Abeysekera, B. (1984) *Phytochemistry* **23**, 951.
17. Ibrahim, R. K. and Grisebach, H. (1976) *Arch. Biochem. Biophys.* **176**, 700.
18. Ibrahim, R. K. (1977) *Z. Pflanzenphysiol.* **85**, 253.
19. Yamamoto, E., Inciong, M. E., Davin, L. B. and Lewis, N. G. (1990) *Plant Physiol.* **94**, 209.
20. Fleuriet, A. and Macheix, J.-J. (1980) *Phytochemistry* **19**, 1955.
21. Shimizu, T. and Kojima, M. (1984) *J. Biochem.* **95**, 205.
22. Rudolph, H., Kirchhoff, M. and Gliesmann, S. (1988) in *Methods in Bryology, Proc. Bryol. Meth. Workshop. Mainz* (Glime, J. M., ed.), p. 25. The Hattori Botanical Laboratory, Nichinan.
23. Rudolph, H. and Rasmussen, S. (1992) *Cryptogamic Botany* **3**, 67.
24. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.
25. Sütfeld, R. (1989) *J. Chromatogr.* **464**, 103.
26. Schwetlick, K. (1970) *Organikum*, Vol. 9. Aufl. VEB Deutscher Verlag der Wissenschaften, Berlin.