Novel Matrices for Biosensor Applications by Structural Design of Redox-Chromogenic Aminocellulose Esters

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ABSTRACT: The solubility and film forming properties of newly developed cellulose derivatives with 1,4-phenylenediamine (PDA) substituents at position C6 of the anhydroglucose unit (AGU) can be achieved by formation of acetate, benzoate, and carbanilate groups, respectively, preferably in position C2/C3. All derivatives, if soluble in *N,N*-dimethylacetamide (DMA) or dimethylsulfoxide (DMSO), form transparent films from their solutions. The solubility of these PDA cellulose esters in DMSO or DMA, such as the aging, enzyme immobilization, and redox-chromogenic behaviors of the films, depending on the nature and the degree of substitution of the functional groups were investigated. Regarding the influence of the autoxidation by air-oxygen of the PDA groups on the storage stability, the longest stable derivatives were the PDA cellulose carbanilates. Onto PDA cellulose derivate films glucose oxidase was immobilized via diazo coupling, and by glutardialdehyde and ascorbic acid reactions with an obtained enzyme activity of 45 (diazo coupling, PDA cellulose acetate) to 145 mU/cm^2 (ascorbic acid, PDA cellulose carbanilate). The redox-chromogenic properties were demonstrated by oxidative coupling reaction of typical reagents like phenol and chinoline derivatives onto the PDA groups of the cellulose esters in presence of H_2O_2 and peroxidase. The best coloring results were obtained by using PDA cellulose carbanilates und benzoates.
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Key words: aminocellulose esters; biosensor support; enzyme immobilization; oxidative coupling; functionalized films

INTRODUCTION

Cellulose derivatives have been used as support for immobilization of different pharmacologically active agents and biomolecules.[1–4] An application is the exploitation as matrix in analyte-sensitive reagent phases of biosensors.[5–7] Such a reagent phase contains a macromolecular support with immobilized biomolecules, e.g., enzymes, for a very specific analyte recognition. The product ("chemical signal") formed during the recognition reaction is transformed into a measurable optical or electron transfer signal by appropriate indicator structures. For this purpose, suitable supports have to contain highly reactive functional anchor groups for immobilizing. Especially for optical sensors, they have to be soluble and to form trans-

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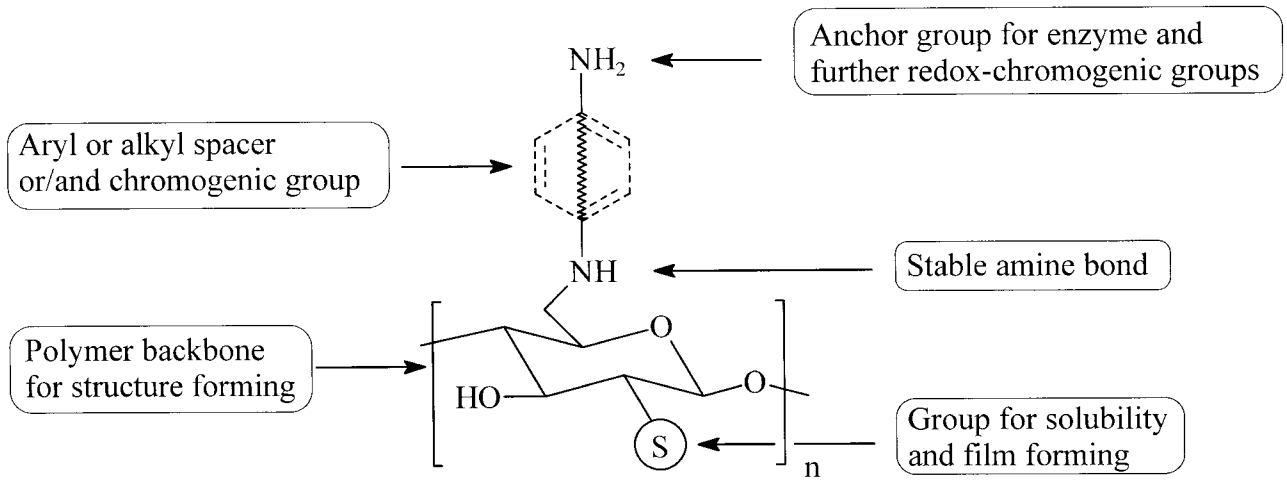

Figure 1 Cellulose derivatives tailor-made for analyte-sensitive reagent phases.

parent films. Moreover, the structures may stabilize the biomolecules for a constant signal flow.

Polyfunctional cellulose structures provide the best prerequisites because of their supramolecular structure-forming potential and because of their biocompatible properties. From this point of view, the aim of our ongoing structural design studies is directed to novel types of cellulose derivatives corresponding to the above requirements. The concept according to Figure 1 consists in the exploitation of the different reactivities of the C2/C3 and C6 hydroxyl groups of the anhydroglucose units (AGU) of cellulose.

In case of reactions with bulky molecules and of S_N2 substitutions the OH function at the C6 position of the AGU is more reactive than the OH groups at C2/C3 caused by the sterical conditions.[8–10] Thus it is possible to obtain regioselective derivatives with anchor groups for immobilizing and units (S) for solubility and film forming. Important parts of such systems are diamino compounds of different spacer types and lengths and redox-chromogenic groups as modified phenylenediamines.

Up to now only one example of soluble and film-forming cellulose derivatives with immobilizing and redox-chromogenic 1,4-phenylenediamine (PDA) groups and *p*-toluenesulfonate (tosylate) units as solutizer has been described.[11,12]

The present communication deals with results on multifunctional cellulose derivatives with PDA groups at the C6 position and with different solutizers (acetate, benzoate, carbanilate), preferably at the C2/C3 atoms of the polymer repeating units. The structural design and the properties of these PDA cellulose esters, such as solubility, film formation, aging, enzyme immobilization, and redox-chromogenic behavior, will be described.

EXPERIMENTAL

Materials

All reagents were of analytical reagent grade. Avicel (microcrystalline cellulose, DP ~ 200) and LiCl obtained from Fluka were dried in vacuum at 100°C for 2 h. Solvents as *N,N*-dimethylacetamide (DMA), *N,N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), and pyridine were dried by CaH_2 and distilled under reduced pressure. Phenyl isocyanate and acetic acid anhydride were distilled before use. The lyophilized glucose oxidase from *Aspergillus niger,* 4-aminoantipyrine, and 2,4,6-tribromo-3-hydroxy benzoic acid were purchased from Boehringer.

Syntheses of the Cellulose Derivatives

p-Toluenesulfonic Acid Esters of Cellulose (Tosylcellulose, 1)

Tosylcellulose was synthesized from Avicel (DP ~ 200) according to McCormick.[20] The DS_{tosylate} was controlled by the molar ratio of *p*-toluenesulfonyl (tosyl) chloride/anhydroglucose unit (AGU) as described.[21] The following four products were prepared: Degree of substitution DS_{tosylate} (in parentheses ratio of mol tosyl chloride/mol AGU): 0.50 (1.5), 1.10 (2.5), 1.50 (3.0), and 1.95 (4.0).

Table I Tosylcellulose Acetates (2), Benzoates (3), and Carbanilates (4) with Different *DS* Values

No.	$DS_{tosylate}$	DS_{acyl}
2a	0.45 (0.50)	2.40
2b	0.95 (1.10)	2.00
2c	1.80 (1.95)	0.80
3a	0.75 (1.10)	2.00
3b	1.10 (1.50)	1.80
4a	0.48 (0.50)	2.30
4b	1.05 (1.10)	1.90
4c	1.88 (1.95)	1.05

For reaction conditions see Figure 2. *DS* values are calculated from elemental analysis; values in parentheses represent $DS_{tosylate}$ before acylation.

6(2)-Tosylcellulose Acetate (Typical Procedure, 2)

Two grams of tosylcellulose ($DS_{tosylate}$ = 0.50, 8.4 mmol modified AGU) dissolved in 10 mL DMF was treated with a solution of 0.8 g anhydrous sodium acetate, 7.8 mL acetic anhydride (84 mmol, 10 equiv/AGU), and 9.0 mL pyridine (103 mmol, 12 equiv/AGU) in 10 mL DMF and stirred at 40°C for 2 days. The product was precipitated in ethanol, filtered, and washed several times with ethanol to remove pyridine. Then it was washed alternately three times with water and ethanol and dried under reduced pressure.

Yield: 2.3 g (83%), $DS_{tosylate}$ 0.45 and $DS_{acetate}$ 2.40 (**2a**, calculated on the basis of elemental analysis, see Table I).

ANAL. Calcd for C, 50.42%; H, 5.25%; S, 4.34%;
Found: C, 49.25%; H, 5.28%; S, 4.38%.

FT-IR (KBr):
3031 (ν, C═C), 2922 (ν, CH_3),
2880 (ν, CH, cellulose), 1598 (ν, C═C),
1454 (ν, C—Ar), 1364 (ν_{as}, SO_2), 1193 (ν, S—Ar),
1177 (ν_{sym}, SO_2), 1061 (ν, C—O—C),
983 (ν, S—O—C); 818 (δ, H—Ar), 2950 (ν, CH_3),
1748 (ν, C═O), 640 (ν, O—C═O),
612 (δ, C═O) cm^{-1}.

^{13}C-NMR (d_6-DMSO):
δ = 20.3–21.1 (CH_3 of tosylate and acetate),
62.0–102.1 (cellulose backbone),
127.1, 129.0, 133.5, and 144.9
(tosylate aromatics),
169.0, 170.3 (C═O) ppm.

6(2)-Tosylcellulose Benzoate (Typical Procedure, 3)

In a modified procedure, according to ref. 15, 2.0 g tosylcellulose ($DS_{tosylate}$ = 1.50, 5.1 mmol modified AGU) was dissolved in 10 DMF and treated with a solution of 4.6 g benzoic acid anhydride (20.4 mmol, 4 equiv/AGU), 3.5 mL pyridine (40.8 mmol, 8 equiv/AGU), and 0.4 g *N,N*-dimethylaminopyridine [10% (m/m) according to benzoic acid anhydride] in 10 mL DMF. Stirring of this mixture was continued at 60°C for 20 h. The product was precipitated in hot ethanol and washed with hot ethanol intensively to remove benzoic acid anhydride. It was then washed quickly with ice-cold water and ethanol three times and dried under vacuum. Yield: 2.3 g (86%), $DS_{tosylate}$, 1.10 and $DS_{benzoate}$, 1.80 (**3b**, calculated on the basis of elemental analysis; see Table I).

ANAL. Calcd for C, 60.86%; H, 4.58%; S, 6.79%;
Found: C, 59.95%; H, 4.74%; S, 6.71%.

FT-IR (KBr):
3031 (ν, C═C), 2922 (ν, CH_3),
2880 (ν, CH, cellulose), 1598 (ν, C═C),
1454 (ν, C—Ar), 1364 (ν_{as}, SO_2),
1193 (ν, S—Ar), 1177 (ν_{sym}, SO_2),
1061 (ν, C—O—C), 983 (ν, S—O—C),
818 (δ, H—Ar), 3063 (ν, C═C),
1731 (ν, C═O), 1599 (Ar),
1580 (Ar), 1280 (ν, C—O),
1110 (ν, C—O—C), 710 (Ar) cm^{-1}.

^{13}C-NMR (d_6-DMSO):
δ = 20.3 (CH_3 of tosylate),
62.0–102.3 (cellulose backbone),
127.0–130.3
(aromatics of benzoate and tosylate),
133.5, 144.9 (aromatic of tosylate),
164.9, 169.6 (C═O) ppm.

6(2)-Tosylcellulose Carbanilate (Typical Procedure, 4)

In a modified procedure according to ref. 13, 10 mL phenyl isocyanate (84 mmol, 10 equiv/AGU) was added to a solution of 2.0 g tosylcellulose (*DS* = 0.50, 8.4 mmol modified AGU) in 20 mL pyridine. Stirring was continued at 70°C for 1 h. The product was precipitated in methanol and washed with methanol to remove pyridine. For removing diphenyl urea, formed by phenylisocyanate with small amounts of water, the product was dissolved in chloroform, and the solution was con-

Table II PDA Cellulose Acetates (5), Benzoates (6), and Carbanilates (7) with Different *DS* Values Prepared from Corresponding Tosylcellulose Esters 2–4

No.	Starting Polymer	DS_{PDA}	DS_{acyl}	$DS_{tosylate}$	Reaction Time (h)[a]
5a	**2a**	0.36	1.88 (2.40)	0.05 (0.45)	16
5b	**2b**	0.56	0.93 (2.00)	0.14 (0.95)	16
5c	**2c**	0.84	0.51 (0.80)	0.59 (1.80)	16[b]
5d	**2c**	0.71	0.11 (0.80)	0.48 (1.80)	20[c]
6a	**3a**	0.35	1.53 (2.00)	0.39 (0.75)	16
6b	**3b**	0.48	1.38 (1.80)	0.49 (1.10)	16
7a	**4a**	0.39	1.74 (2.30)	0.05 (0.48)	16
7b	**4b**	0.57	1.36 (1.90)	0.17 (1.05)	16
7c	**4c**	0.38	0.92 (1.05)	1.22 (1.88)	1
7d	**4c**	0.76	0.86 (1.05)	0.71 (1.88)	3
7e	**4c**	0.91	0.74 (1.05)	0.41 (1.88)	16

DS values were calculated from elemental analysis; values in parentheses represent the *DS* values before reaction with PDA (cf. Table I).

[a] General conditions: DMSO, TEA, molar ratio PDA/tosylate group = 8 : 1, 100°C.

[b] The reaction was carried out in a DMSO/DMA mixture (v/v, 1 : 1).

[c] The reaction was carried out in DMA.

centrated to oil under reduced pressure. With the cooling of the residue, diphenyl urea crystallized. The product was dissolved in chloroform, the diphenyl urea crystals were filtered off, and the solution was concentrated again. This procedure must be repeated as long as diphenyl urea crystallizes. The final product was precipitated in ethanol, washed with ethanol, and dried under reduced pressure. Yield: 3.0 g (70%), $DS_{tosylate}$, 0.48 and $DS_{carbanilate}$, 2.30 (**4a**, calculated on the basis of elemental analysis, see Table I).

ANAL. Calcd for C, 59.96%; H, 4.78%; N, 6.32%;
S, 3.02%;
Found: C, 59.14%; H, 4.82%; N, 6.45%; S, 2.92%.

FT-IR (KBr):
3058 (ν, C═C), 2922 (ν, CH_3),
2880 (ν, CH, cellulose), 1748 (ν, C═O),
1598 (ν, C═C), 1532 (ν, C—N—H),
1444 (ν, C—N), 1364 (ν_{as}, SO_2), 1217 (ν, C—O),
1193 (ν, S—Ar), 1177 (ν_{sym}, SO_2),
1061 (ν, C—O—C), 983 (ν, S—O—C),
818 (δ, H—Ar), 756 (Ar) cm^{-1}.

^{13}C-NMR (d_6-DMSO):
δ = 20.3 (CH_3 of tosylate),
59.1–101.8 (cellulose backbone),
119.1–144.9
(aromatics of carbanilate and tosylate),
152.8, 153.6 (C═O) ppm.

PDA Derivatization of 2–4 (General Procedure, 5–7)

One gram of a tosylcellulose ester was dissolved in 10 mL DMSO, and 8 mol PDA per tosylate group was added under shaking at room temperature. After addition of 5 mL triethylamine, shaking was continued at the temperature of 100°C for 0.5–16 h, depending on the preferred DS_{PDA} of the product. The polymer was precipitated in 100 mL ethanol, filtered off, and washed with ethanol for at least five times. The filtrate was examined for traces of PDA by adding phenol and H_2O_2 (30%, v/v). Washing was finished if no more coloring occurred. The product was dried under reduced pressure at room temperature for 6 h. Yield: 50% to 70%. The *DS* values based on elemental analysis are shown in Table II.

Appearing new signals:

FT-IR (KBr):
3380 (ν, NH_2), 3031 (ν, C═C),
1517 (Ar), 1260 (ν, N—C) cm^{-1}.
NMR: 114.1, 115.4, 139.7, 140.3
(PDA aromatics) ppm.

Film Formation

A glass rod with a diameter of ca. 1.3 mm was dipped briefly into a 5% solution of PDA cellulose ester in DMA (immersion depth ca. 5 mm) and

Table III GOD Activity Coupled on Films of PDA Cellulose Esters Using Different Immobilization Methods

Immobilization Method	GOD Activity (mU/cm^2) Obtained for the Polymers[a]		
	5a (0.36)[b]	**6b** (0.48)	**7a** (0.39)
Glutardialdehyde reaction	120	105	135
Diazo coupling	45	120	90
Ascorbic acid reaction	130	110	145

See Figure 2 and Table II.
[a] 1 Unit (U) = conversion of 1 μmol substrate per 1 min.
[b] The values in parentheses are the DS_{PDA} of the PDA cellulose esters.

subsequently air-dried. A colorless, uniform, and ultrathin film with an area of ca. 0.2 cm^2 was formed on the glass surface. This film was used for all investigations with respect to enzyme immobilization and chromogenic properties.

Enzyme Immobilization Procedures

For all immobilizations an aqueous solution of glucose oxidase (GOD; 60 U/mL) was used.

Method A: Glutardialdehyde Activation

For activation purposes, the polymer films on the glass rod described above are swollen in DMA for about 10 s and then immersed in a 20% aqueous glutardialdehyde solution for 30 min. The film is subsequently washed five times with water, immersed in the enzyme solution, and incubated at 4°C for 12 h.[16]

Method B: By Means of Diazo Coupling

The polymer film was swollen in DMA and subsequently placed in 10 mL of ice-cooled 0.5*M* HCl. One milliliter of 1*M* $NaNO_2$ solution was added dropwise while the film was cooled to 4°C. After a contact time of 5 min, the yellow-stained polymer film was rinsed rapidly several times with water, placed in the enzyme solution, and incubated at 4°C for 12 h.[17]

Method C: Ascorbic Acid Activated

The polymer films are dipped in a concentrated solution of ascorbic acid in DMA/ethanol (1 : 5) and incubated until the film is swollen visibly. With respect to solvent and support, the reaction time is in the range of 1 to 30 min. After incubation the films are washed three times with water and immersed in the enzyme solution for 16 h.[18]

Determination of GOD Activity

After complete removal of adhering enzyme solution by rinsing the film with distilled water, the immobilized GOD activity was determined photometrically using a UV/VIS spectrophotometer (Beckman DU-64). For this purpose, the rod with the enzyme-linked film was placed in a cuvette containing a solution of the test system. The activity measurements were carried out at 25°C while the solution was stirred. The test system used is a modified test after Trinder[22] and contains 4-aminoantipyrine and 2,4,6-tribromo-3-hydroxybenzoic acid dissolved in phosphate buffer, pH 7.0. The increase of absorbance is measured at 514 nm (absorption coefficient ε = 27.2 cm^2/μmol). The enzyme activities obtained are listed in Table III.

Oxidative Coupling Procedure

The films of the PDA cellulose esters were formed on glass rods as described above. The rods were immersed in 1 mL of a 10 m*M* ethanolic solution of the coupling reagent (see Table IV) for 10 min. In this time the films swell slightly. A mixture of 3 U (0.01 mg) peroxidase, 0.01 mL of a 100 m*M* H_2O_2 solution, and 1 mL phosphate buffer, pH 8.0, was added to the solution. The films were stained within 10 s to 5 min, depending on the

Table IV Absorption Maxima λ_{max} of Cellulose Esters Colored by Oxidative Coupling of Typical Reagents with Different PDA Cellulose Esters

Coupling Reagent	Starting Polymer		
	5a	**6b**	**7a**
Phenol	585	589	608
2,4-Dichlorophenol	590	580	608
2,3-Dichlorophenol	608	626	641
2,4,5-Trichlorophenol	616	617	641
2,4,6-Trichlorophenol	635	642	653
2,4-Dibromophenol	620	614	631
5-Chloro-8-hydroxychinoline	575	576	592
5,7-Dibromo-8-hydroxychinoline	606	600	611
Gallic acid methylate	605	601	615

Values (λ_{max}) are expressed in nanometers.

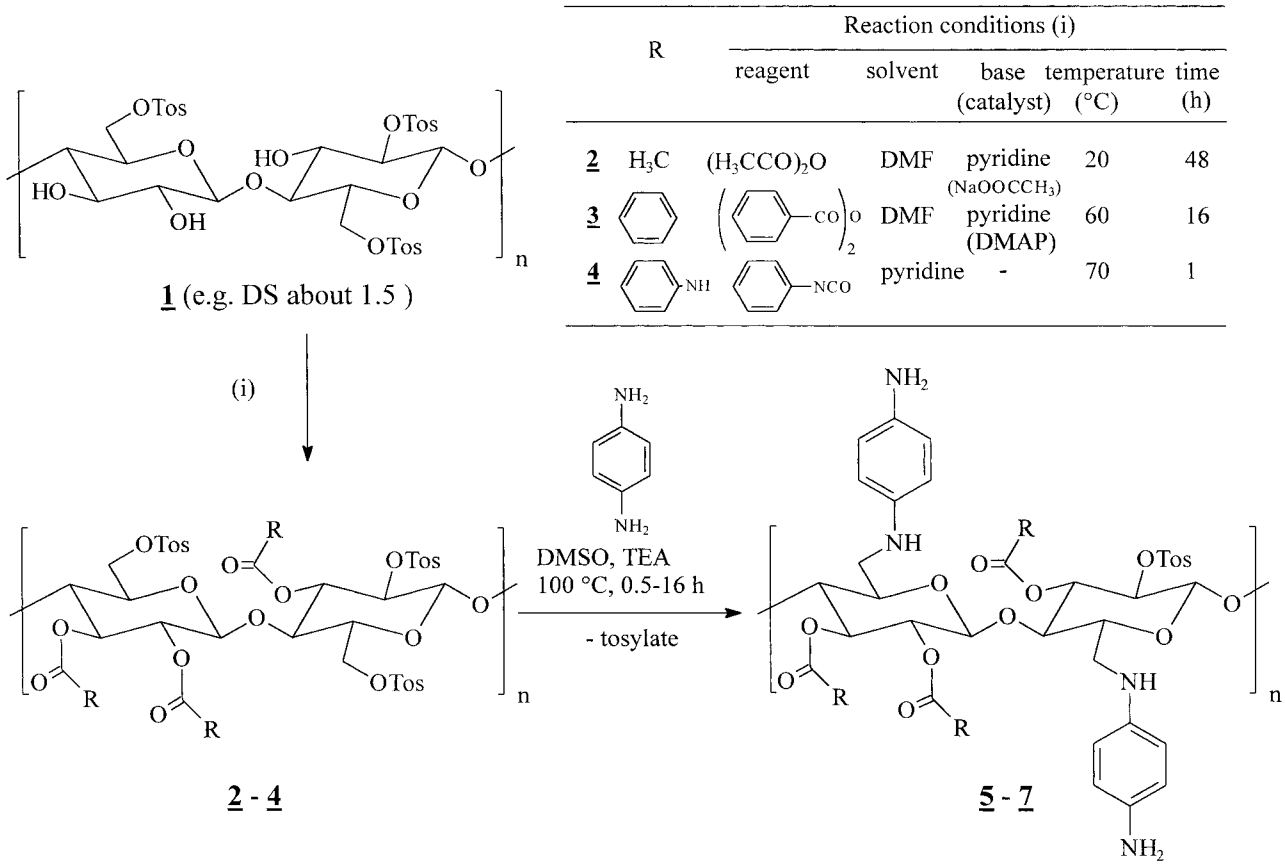

Figure 2 PDA cellulose esters by subsequent reactions of tosylcelluloses (*DS* values and corresponding structure details see Tables I and II).

coupling reagent. The colored film was washed with buffer, pH 8.0, and distilled water, and dried in air. The film was subsequently measured by fiber optics. The glass rod was positioned between two fiber-optic devices and measured by a UV/VIS spectrophotometer (X-dap, Polytec IKS-optoelectronics, Waldbronn and Duisberg, Germany) equipped with a fiber-optic inlet and outlet. The absorption maxima λ_{max} are listed in Table IV.

Measurements

The *DS* values were determined on the basis of sulphur, nitrogen, and hydrogen content of elemental analysis. The FT-IR and ^{13}C-NMR spectra were recorded on a Nicolet IMPACT 400 Fourier transformation spectrometer (Madison, WI) and on a Bruker AMX 400 spectrometer (Billerica, MA), respectively.

RESULTS AND DISCUSSION

Syntheses and Structural Design

The PDA cellulose esters (**5–7**, see Fig. 2 and Table II) were synthesized by nearly complete acylation of the free OH groups of *p*-toluenesulfonic acid esters of cellulose (tosylcelluloses **1**; *DS* 0.5–1.9),[13] followed by a substitution reaction of tosylate groups in the polymers **2–4** (see Table I) with PDA as shown in Figure 2.

The substitution of tosylate groups by PDA is difficult because of the cleavage of ester groups in the presence of amines as a side reaction. In preliminary experiments cellulose triacetate, tribenzoate, and tricarbanilate were treated with aliphatic (*n*-butylamine) and aromatic (PDA) amines under typical reaction conditions of the tosylate substitution. As demonstrated by IR spectroscopy and elemental analysis only acetyl groups are cleaved by aminolysis in case of *n*-butylamine. No cleavage occurs by using PDA because of its lower basicity and nucleophilicity.

To examine and to design the properties of the synthesized multifunctional PDA cellulose esters depending on the degree of substitution (ratio: DS_{PDA}/DS_{acyl}), systematically modified tosylcelluloses from low (*DS*, 0.50) up to high (*DS*, 1.95) degree of tosylation were used. The corresponding

$DS_{tosylate}$ values could be achieved by varying the molar ratio of *p*-toluenesulfonyl chloride (tosylchloride)/AGU in the tosylation step. The acetylated, benzoylated, and carbanilated polymers (according to Fig. 2) are summarized in Table I.

The tosylcelluloses were acetylated as described.[13] The IR spectra of acetates **2a–c** show no significant signal between 3200 and 3500 cm^{-1}, which means that nearly all remaining OH groups of the tosylcellulose were acylated. The C═O valency vibration appears at 1748 cm^{-1}. Different from the literature,[13] the benzoylation was carried out with benzoic acid anhydride, pyridine, and DMAP in DMF at 60°C for 16 h. Under these conditions, nearly complete esterification of the free OH groups (DS_{total}, 2.9) occurs. In the IR spectra, the C═O valency vibration of **3a,b** appears at 1731 cm^{-1}. In contrast to acetylation, under the conditions of benzoylation, cleavage of the tosylate groups up to 30% was observed. The preparation of the tosylcellulose carbanilates **4a–c** was carried out using 10 mol phenylisocyanate/mol modified AGU. A nearly quantitative yield with a DS_{total} of 2.8–2.9 could be obtained after reaction at 70°C for 1 h. Characteristic FT-IR absorptions for the carbanilate group were found at 3375 (ν, N—H) and at 1747 cm^{-1} (ν, C═O).

While the solubilities of the tosylcellulose benzoates and carbanilates did not change with different $DS_{tosylate}$, the tosylcellulose acetates were less soluble with increasing $DS_{tosylate}$ values. Therefore, a highly substituted tosylcellulose ($DS_{tosylate}$ = 1.95) is insoluble in DMSO after acetylation.

The reaction of the tosylcellulose esters **2–4** (except **2c**) with PDA was carried out in DMSO at 100°C in the presence of triethylamine as described.[11]

A substitution of all tosylate groups by PDA has never occurred in all studied cases. In the ^{13}C-NMR spectra of all PDA cellulose derivatives, the former peak of free PDA at δ = 114 ppm is split into two signals (114.1 and 115.4 ppm), which is characteristic of the covalent bonding of PDA on cellulose.

The PDA cellulose esters formed under different reaction conditions are listed in Table II. The substitution of the tosylate groups by PDA proceeds after an S_N2 mechanism. Because of sterical hindrance the formation of a transition complex is only possible in position C6 of the AGU and therefore only primary tosylate groups (tosylate groups at C6) are substituted. This corresponds to the results of other substitution reactions with tosylcellulose, e.g., the reaction with phenolates and halides. In these reactions $DS > 1$ was not reached.[8,14] In case of PDA as reagent about 9–15% of the primary tosylate groups are split off by aminolysis. The time-dependent increase of DS_{PDA} was investigated using the samples **4a–c** (Fig. 3).

It can be seen that the substitution of the tosylate groups by PDA occurred up to more than 90% after 6 h. Further reaction only leads to cleavage of the secondary tosylates as well as the carbanilate groups without increase in DS_{PDA}. Only with **4c** ($DS_{tosylate}$ 1.88) DS_{PDA} of nearly 1.0 (DS_{PDA} 0.91) could be achieved. For $DS_{tosylate}$ of 1.05 (**4b**) and of 0.48 (**4a**), respectively, 57% (DS_{PDA} 0.57) and 87% (DS_{PDA} 0.39) of tosylate groups are substituted by PDA. Thus, with a lower $DS_{tosylate}$, a higher percentage of tosylate groups could be substituted by PDA.

The maximum DS_{PDA} for acetates was 0.84 (starting from **2c**). Because of the cleavage of tosylate groups during the benzoylation, the maximum DS_{PDA} of 0.48 starting from **3b** is lower than the obtained DS_{PDA} for a comparable tosylcellulose carbanilate (**7b** from **4b**). The cleavage of the ester groups, during the reaction with PDA in dependence on the reaction time, was studied in case of the polymers **2b**, **3b**, and **4b**. In Figure 4 the time course of the relative content of ester groups [$DS(t)_{acyl}/DS(t_0)_{acyl}$] is shown.

It can be seen that an obvious cleavage of the ester groups occurs during the reaction. As expected, the acetate residue is most unstable (loss of 54% after the reaction time of 16 h). In the same time, 24% of the benzoate groups and 30% of the carbanilate groups were split off. In all cases the highest cleavage rate was observed at the beginning of the reaction.

We suppose that the ester groups were split off by aminolysis by PDA and TEA, respectively. Because of the insolubility of the tosylcellulose acetate **2c** ($DS_{tosylate}$ 1.80, $DS_{acetate}$ 0.80) in DMSO, the reaction with PDA was performed in a mixture of DMSO/DMA (1 : 1) (**5c**) and in DMA (**5d**), respectively. With increasing content of DMA in the reaction mixture the cleavage of the acetate groups increases, too, presumably because of traces of dimethylamine in the DMA.

Solubility, Film Formation, and Aging by Autoxidation

All soluble PDA cellulose esters form colorless and transparent films from their solution in DMA

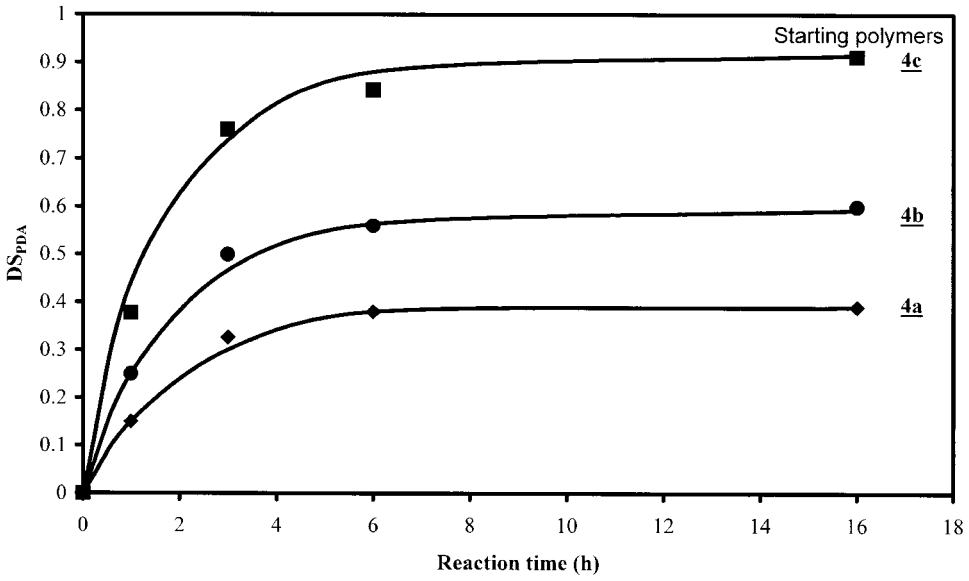

Figure 3 Substitution of the tosylate groups of the tosylcellulose carbanilates (**4a**, **4b**, and **4c**) by PDA depending on the reaction time; general conditions: DMSO, TEA, molar ratio PDA/tosylate groups = 8 : 1, 100°C.

or DMF in each ratio of DS_{PDA}/DS_{acyl}. These films are mechanically more stable and less brittle than those of the PDA celluloses without further solutizer groups, except tosylate groups, described up to now.[11]

The free form of PDA is autoxidable by air–oxygen.[19] The autoxidation tendency decreases if PDA is bonded onto cellulose. The autoxidation of the PDA groups was investigated in films and DMA solutions of the PDA cellulose esters. From the films (stored in air at room temperature) and the solutions (stored at 4°C) UV/VIS spectra were recorded daily. The increasing absorption at 420 nm is a measure for the degree of oxidation. Fig-

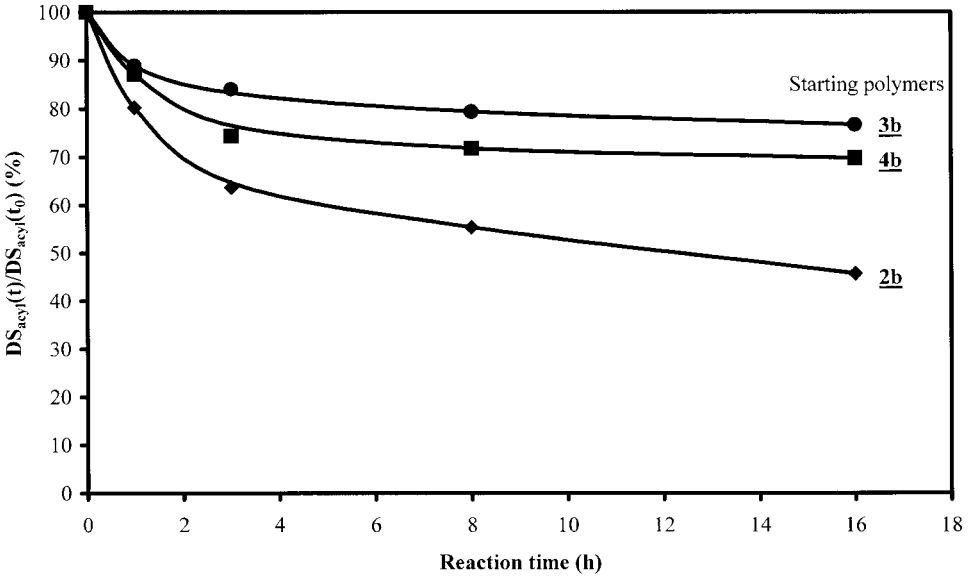

Figure 4 Cleavage of ester groups during the reaction of tosylcellulose acetate (**2b**), benzoate (**3b**), and carbanilate (**4b**) with PDA (molar ratio PDA/tosylate groups = 8 : 1) in DMSO in the presence of TEA at 100°C, depending on the reaction time.

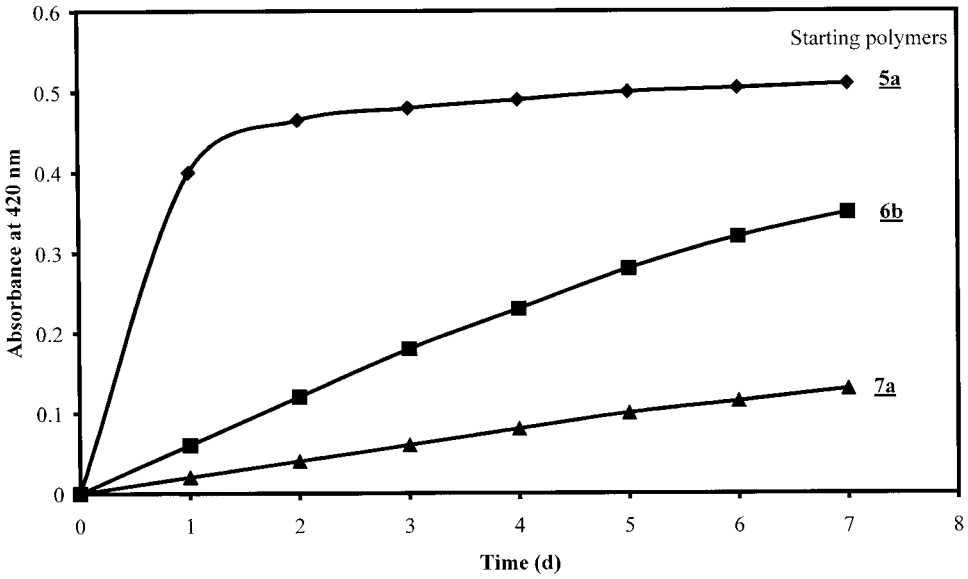

Figure 5 Time course of oxidation of the PDA groups in films of the PDA cellulose acetate (**5a**), benzoate (**6b**), and carbanilate (**7a**) (for *DS* values see Table II) measured by UV/VIS spectroscopy at 420 nm.

ure 5 shows the typical increase of absorbance at 420 nm for films of the polymers **5a**, **6b**, and **7a**.

As can be seen in Figure 5, the PDA groups of the acetates (**5a**) are oxidized within 2 days. The benzoate **6b** is more stable. The best stability against oxidation shows the carbanilate **7a**. We assume that the stabilization of the PDA group is caused by the formation of an intracellular hydrogen bond between the NH unit of the urethane bond of the carbanilate and the secondary amino function of the PDA group. Comparable results were obtained for DMA and DMSO solutions of the PDA cellulose derivatives. Generally, the staining rate increases with increasing DS_{PDA}.

There are three main factors influencing the solubility of PDA cellulose derivatives **5–7**. At first, the amino groups of the PDA unit and the residual OH groups of the AGU can form hydrogen bridges. Second, the NH_2 function of PDA may react with further primary tosylate residues at the AGU. Third, the PDA groups may be converted by autoxidation into the corresponding diimine, which forms different products by oxidative coupling with other PDA units, such as the Bandrowski base.[15] The last two reactions lead to a crosslinking that is irreversible.

All obtained PDA cellulose esters are soluble in DMSO and DMA after isolation. The solubility of these cellulose derivatives in DMA, depending on the storage time, is shown in Figure 6. Best results were obtained by the PDA cellulose carbanilates. Generally, the solubility decreases with increasing DS_{PDA} because of crosslinking by formation of hydrogen bridges and higher autoxidation tendency. Surprisingly even low DS_{acyl} are necessary to improve the solubility of PDA cellulose esters. For example, **5d** ($DS_{acetate}$ 0.11) is soluble in DMA after the storage time of 1 week. A comparable PDA cellulose (DS_{PDA} 0.75, $DS_{tosylate}$ 1.30) is insoluble after drying. PDA cellulose carbanilates with residual primary tosylate groups (**7c,d**) are insoluble in DMA and DMSO after a short storage time, depending on DS_{PDA} and $DS_{tosylate}$. The carbanilate **7c** (from **4c**) with $DS_{PDA} < 0.5$ is insoluble in DMA after 2 months' storage. The carbanilates (e.g., **7d** from **4c**) with DS_{PDA} between 0.5 and 0.8 lose their solubility in DMA after 3 weeks, obviously because of crosslinking by substitution reactions. With $DS_{PDA} > 0.8$ the carbanilates (e.g., **7e**) are still soluble in DMSO and DMA even after 24 weeks. Within a few months only a pale brown coloring occurs in the case of these derivatives.

PDA cellulose acetates and benzoates turn to brown in the time of few days, but they are still soluble after several weeks. Obviously, the crosslinking processes based on the oxidation of the PDA groups are responsible for the insolubility of **5** and **6** after short storage time. The benzoates have better solubility properties than acetates because of the lower autoxidation rate (see Fig. 5).

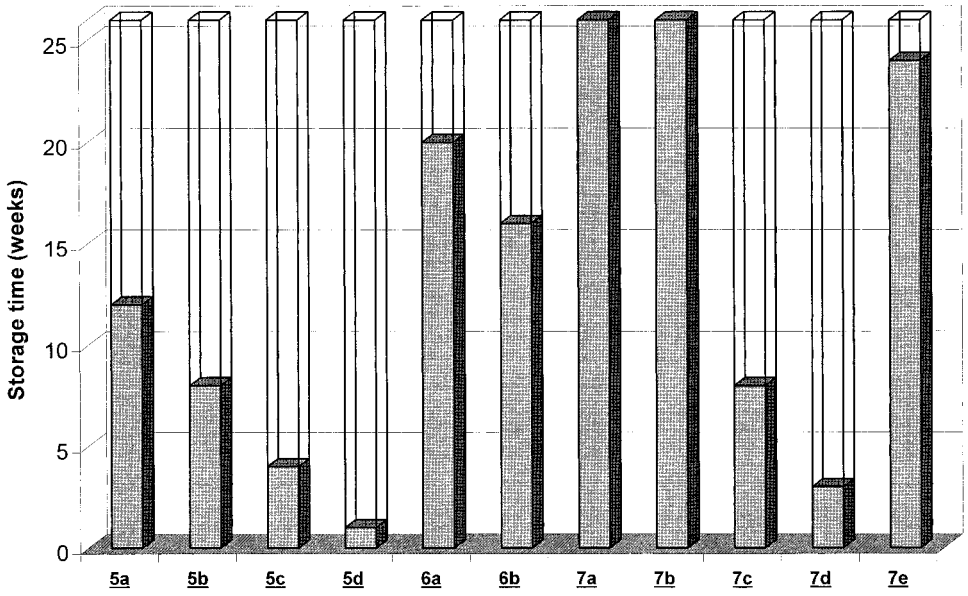

Figure 6 Solubility of different PDA cellulose acetates (**5**), benzoates (**6**), and carbanilates (**7,** cf. Table II) in DMA in dependence on the storage time. The filled part of the column means that 5 mg of the certain derivative is soluble in 1 mL DMA.

Enzyme Immobilization

The enzyme immobilizing potential of the PDA cellulose esters was studied with glucose oxidase (GOD) as an example. For film forming a glass rod is dipped in a 5% DMA solution of typical PDA cellulose acetate (**5a**), benzoate (**6b**), and carbanilate (**7a**), respectively, and dried in air at room temperature. GOD was immobilized on these films using the well-known surface activation by means of glutardialdehyde reaction,[16] diazo coupling,[17] and the novel ascorbic acid reaction.[18] To activate the films before treatment with the immobilizing reagents, they were swollen in a mixture of ethanol/DMSO (v/v, 5 : 1 up to 20 : 1) for 1–5 min. The rate of swelling decreases with storage of the layers. For reproducable results, the samples were used 4 h after preparation. The enzyme immobilization was carried out at 4°C overnight. The enzyme loading was defined as the enzyme activity per area (mU/cm^2). After washing, the immobilized GOD was measured by stirring the enzyme-linked layer in a solution of a photometrical test system. To determinate only covalently immobilized enzyme, the procedure was repeated until the signal release into the test solution was $< 1\%$ of the obtained total value. The immobilized GOD activities obtained for typical PDA cellulose esters are listed in Table III.

Surprisingly, the GOD activities immobilized on PDA cellulose esters with various ester groups are in the same range for most samples, i.e., the ester groups do not have a decisive influence on the enzyme immobilization. The coupled GOD activity is stable by storage in water at 4°C. Even after 4 weeks the GOD activities amounted to 85–95% of their starting values. Thus, the PDA cellulose esters are excellently suited for enzyme immobilization. Furthermore, films from PDA cellulose acetate and benzoate can be activated by swelling in a 1% (v/v) *n*-butylamine/H_2O solution. The swelling of the hydrophobic layers may be explained by an increase of hydrophilicity caused by partial deacylation. However, this preliminary treatment of the films does not lead to an increase in immobilizable GOD activity.

Redox-Chromogenic Properties

With respect to the use of PDA cellulose esters as support for analyte-sensitive reagent phases in fiber-optical biosensors, the introduction of redox-chromogenic groups, which serve as "optical signal units," is essential. The PDA group is especially suited because of a variety of such signal structures, covalently bound to the polymer, can be produced by a peroxidase (POD)-catalyzed ox-

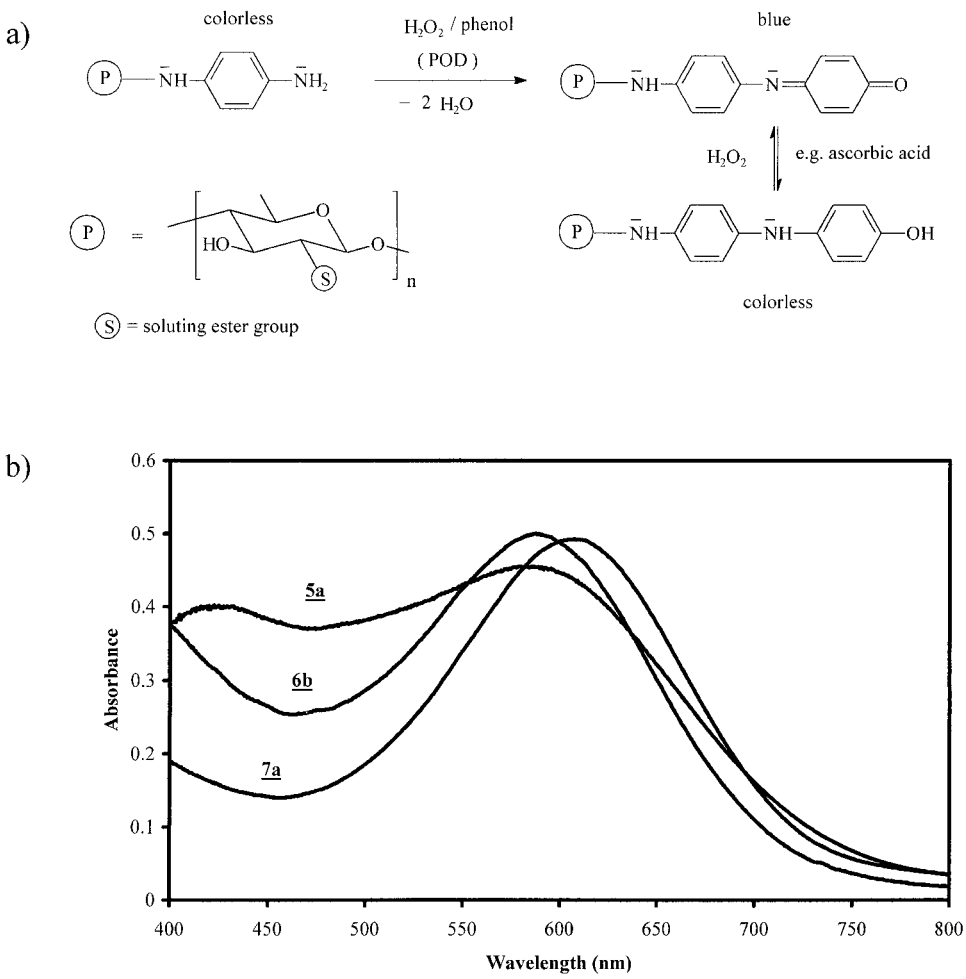

Figure 7 Principle of coloring by the oxidative coupling of phenol onto PDA cellulose esters (a) and the VIS spectra (b) of the phenol-coupled PDA cellulose acetate (**5a**), benzoate (**6b**), and carbanilate (**7a**).

idative coupling (see Fig. 7).[15] To study the influence of the different solutizing ester groups on the degree of formation of redox-chromogenic structural units and on the properties of the chromogenic polymers formed, nine different reagents were oxidatively coupled onto the PDA cellulose derivatives. This oxidative coupling reaction is started by oxidation of the PDA group by H_2O_2 to the corresponding, cationic diimine, which can form the coupling product or the uncharged diimine and following products,[19] not capable for coupling. Therefore, the oxidation rate of the PDA group and the rate of coupling with the cationic quinonediimine group is significant for the reaction course.

The VIS spectra of the films of PDA cellulose acetate, benzoate, and carbanilate colored by using phenol are shown in Figure 7.

PDA cellulose acetate shows an additional absorption at 420 nm, indicating a high content of oxidized PDA (Fig. 7). For PDA cellulose carbanilate (**7a**) and benzoate (**6b**) no strong absorption was detected in that region. Therefore, it could be concluded that the oxidative coupling with these derivatives occurs in higher yield, obviously because of the lower oxidation rate of the PDA groups. The same effect was found for all used coupling reagents. The absorption maxima of typical examples of colored cellulose esters formed are listed in Table IV.

The absorption maxima of the colored PDA cellulose acetate (**5a**) and benzoate (**6b**) are shifted to lower wavelengths compared to the carbanilate (**7a**). This effect probably appears because of the acidic conditions in the cellulose films due to hydrolysis of the ester groups. The colored products

can be reduced to the colorless (leuco) form using a solution of ascorbic acid in ethanol. A subsequent coloring by oxidation with 10–100 m*M* aqueous H_2O_2 is possible within 1–10 min.

Because of their redox-chromogenic properties the described PDA cellulose esters are suited for the use in optical detection systems as well as in water-resistant colored layers.

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