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Note

Coupling of hydroxycinnamic acids to epoxy-activated Sepharose 6B

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Epoxy-activated agarose gel is a suitable matrix support for affinity chromatography because of the stability of the ether link between the gel and the hydrophilic spacer. Usually, ligands bearing amino groups have been coupled with agarose[1–3]. Gelsema *et al.*[4] optimized the reaction of the aromatic amine on the epoxy ring. Using alcohols, Sundberg and Porath[1] studied the reaction of agarose bearing hydroxyl groups with diglycidyl ether. However, there have been few reports on the coupling of alcohols with epoxy-activated agarose. Vretblad[5,6] added sugars to the oxirane group without studying the reactivity of different hydroxyl groups.

In this paper, we describe the optimization of the coupling of 2-hydroxy- and 3-hydroxycinnamic acids, via a phenol side group, with epoxy-activated agarose gel. First, to clarify the behaviour of the system, the addition of hydroxycinnamic acid on an epoxy ring was taken as a model: the most representative model for our system was 2,3-epoxypropyl isopropyl ether. Then, the same reaction was carried out on the agarose gel and the influences of four parameters were studied: reaction time, sodium hydroxide and ligand concentrations, temperature.

MATERIALS AND METHODS

Reagents

o-Hydroxy- and *m*-hydroxycinnamic acids (Aldrich) and 2,3-epoxypropyl isopropyl ether (Merck) were used without further purification.

Epoxy-activated Sepharose 6B (Pharmacia Fine Chemicals) was swollen on a glass filter for 30 min and washed with distilled water. The gel used contained 20 μmol/ml of epoxy groups.

Model reaction: synthesis of 2-(2-hydroxy-3-isopropoxypropoxy)cinnamic acid

A 1-g (6.1-mmol) amount of 2-hydroxycinnamic acid was dissolved in a 2 *M* sodium hydroxide solution containing 2 mg/ml of sodium tetrahydroborate. 2,3-Epoxypropyl isopropyl ether (0.71 g, 6.1 mmol) was added. The yellow mixture was heated to 50°C for 30 h. The resulting solution was acidified and extracted with diethyl ether. The product was purified by chromatography silica gel (yield 56%).

IR spectra were recorded on a Perkin-Elmer 983 spectrophotometer using tetrachloromethane as the solvent. ^{13}C NMR spectra were recorded on a Brucker 50 MHz instrument using deuterochloroform as the solvent and tetramethylsilane as the

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internal standard. Gas chromatographic (GC) measurements were performed on a Varian 3400 chromatograph with an OV-17 column. A Ribermag R 10 LOB instrument was used for the mass spectrometry. The obtained products were silylated with N,O-bis(trimethylsilyl)trifluoroacetamide.

Coupling conditions

Swollen gel samples (1.5 ml) were mixed in a 25-ml round-bottom flask with hydroxycinnamic acid in sodium hydroxide solution containing 2 mg/ml of sodium tetrahydroborate. The mixture was then incubated for a given time at a set temperature on a shaker in a water-bath. The gel was then poured into 100 ml of water, allowed to stand for 30 min, filtered, washed with a large excess of water and stored at 8°C.

Determination of the degree of hydroxycinnamic acid substitution

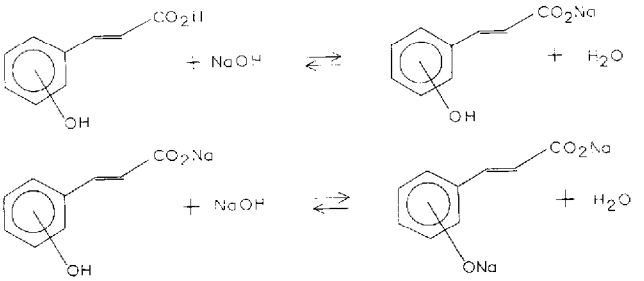
Aliquots (50 mg) of the grafted gel were suspended in 2 ml glycerol and UV spectra were recorded in a 2-mm cell with a MPS 2000 spectrophotometer. The hydroxycinnamic acid content was calculated from the absorbance at 275 nm for the *meta* isomers and that at 280 nm for the *ortho* isomers.

RESULTS AND DISCUSSION

Model reaction

Usually the reaction between the epoxy ring and a nucleophilic group is performed in an aprotic solvent such as dimethylformamide (DMF), dioxane[7,8], etc. However, our strategy was to use water, in which the gel swells better than in organic solvents. Indeed, the bed volume[9] of an epoxy-activated gel is 3 ml in water, as compared to 2.5 ml in DMF.

Scission of the oxirane group can occur under both acidic and basic conditions. In the present case, the acetal structure of agarose renders the use of an acidic medium unfavourable. So we worked in sodium hydroxide aqueous solution. Hydroxycinnamic acid has two reactive functions, an alcohol and an acid, which can react with two equivalents of sodium hydroxide as follows:



The epoxy ring can be opened by phenoxide or carboxylate ions[13] and is hydrolyzed according to the routes shown in Fig. 1. The hydrolysis of the epoxy ring gives compound D. The addition of carboxylate[6,10] ions to the epoxy ring should result in compound A. Compounds B and C result from attacks on the epoxy ring by mesomeric forms of phenoxide ions (see Fig. 2). In a protic solvent such as water

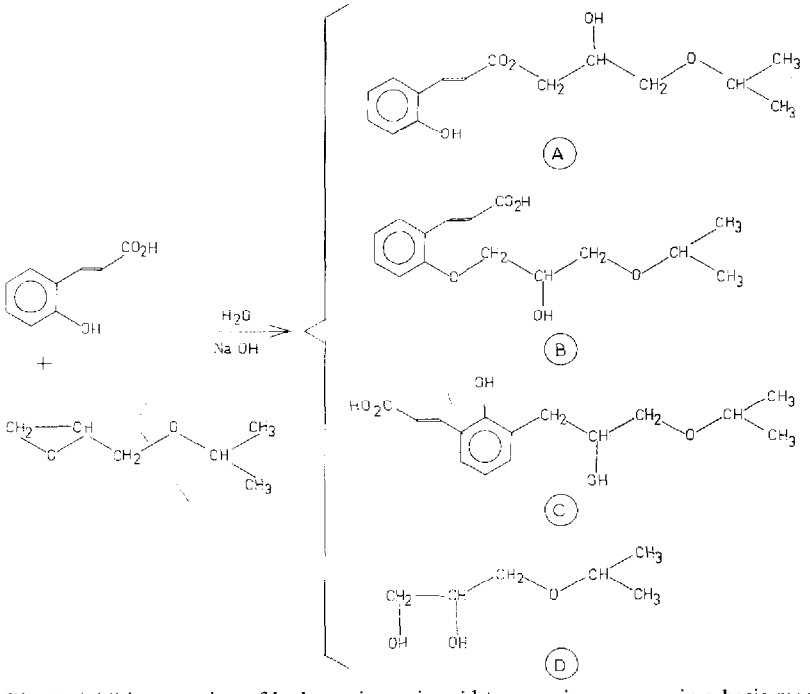


Fig. 1. Addition reaction of hydroxycinnamic acid to an oxirane group in a basic medium.

or methanol, phenolic salts exist in mesomeric forms which can result in O-alkylation and C-alkylation[11].

Analysis of the products obtained. The IR spectrum of the products exhibits only one strong band at 1689 cm^{-1}, corresponding to the carbonyl stretching vibration range, typical of a carboxylic acid group. No trace of an ester group was observed, implying the absence of compound A. In the ^1H NMR spectrum, there are no signals, characteristic of epoxy rings (δ = 2.5–2.7 and 3 ppm); thus, these rings have all been opened.

GC shows three compounds which are characterized by mass spectrometry as B, C and D. Table I presents the typical mass peaks of compounds B and C after silylation by N,O-bis(trimethylsilyl)trifluoroacetamide. The major compound observed is B and there is only a trace of compound C.

The ^{13}C NMR chemical shifts of compound B are in good agreement with values valculated from chemical shifts given by Ewing[12] (see Table II).

The study of this reaction has led to the following conclusions: the reaction is possible in water at 50°C; the carboxylate ion is not effective in the opening of the epoxy ring; the desired product B is obtained in 56% yield because of the competition between hydroxide and phenoxide ions for ring opening.

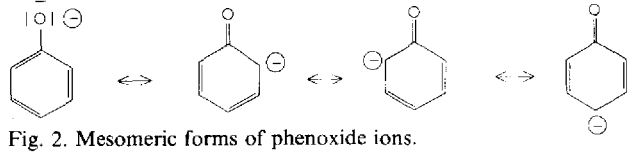


Fig. 2. Mesomeric forms of phenoxide ions.

TABLE I

MASS SPECTROMETRY OF O- AND C-ALKYLATED PRODUCTS AFTER SILYLATION

Product	M^+	*Characteristic peaks*
O-alkylated	*m/e* 424	*m/e* 366, 278, 249, 146, 112, 102
C- alkylated	*m/e* 496	*m/e* 461, 423, 406, 352, 129, 117

Grafting of the gel

Due to the competition between hydrolysis and the addition of phenoxide ions to the epoxy ring (Fig. 3), we tried to optimize the coupling of hydroxycinnamic acid to the gel. The influences of the following parameters on the yield were studied: reaction time, sodium hydroxide and hydroxycinnamic acid concentrations, temperature.

In the following reactions, an excess of sodium hydroxide with respect to hydroxycinnamic acid was used. In the discussion, the following terms will be used

$$X = \frac{[\text{NaOH}] - [\text{hydroxycinnamic acid}]}{[\text{hydroxycinnamic acid}]}$$

$$Y = \frac{[\text{hydroxycinnamic acid}]}{[\text{epoxy}]}$$

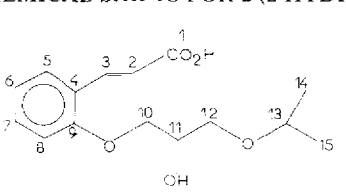
where [NaOH] is the sodium hydroxide concentration, [hydroxycinnamic acid] is the hydroxycinnamic acid concentration and [epoxy] is the epoxy ring concentration, and Z = Reaction yield · 100

The reaction yield is expressed as the ratio between the amount of hydroxycinnamic groups introduced in the gel and the epoxy ring concentration at the beginning of the reaction.

Optimum reaction time. This study was carried out at 30°C, using 500-mg gel samples and $X = 1$, $Y = 10$. Fig. 4 shows the reaction yield as a function of time.

TABLE II

^{13}C NMR CHEMICAL SHIFTS FOR 2-(2-HYDROXY-3-ISOPROPOXYPROPOXY)CINNAMIC ACID



	C^1	C^2	C^3	C^4	C^5	C^6	C^7	C^8	C^9	C^{10}	C^{11}	C^{12}	C^{13}	C^{14}	C^{15}
Calculated				122	129.03	121.03	131.6	114.37	158.22						
Found	171.99	118.27	142.06	123.70	129.25	121.34	132.00	112.76	157.81	69.92	69.34	69.05	72.61	22.13	22.13

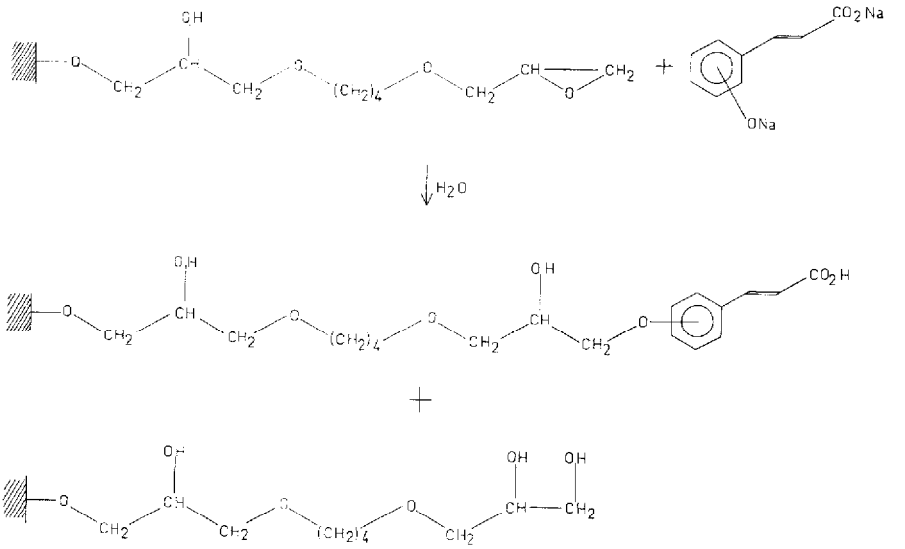


Fig. 3. Coupling of hydroxycinnamic acid to epoxy-activated Sepharose 6B.

It was verified that all the epoxy rings were opened at the end of the reaction. The maximum yield was reached more quickly for the *meta* isomer (28 h) than for the *ortho* isomer (32 h). However, the optimum yield was found to be higher for the *ortho* isomer. These results can be explained by the respective concentrations of the different phenoxide isomers and free hydroxide ions (equilibria I and II). For a given value of X, the concentration of the *meta*-phenoxide isomer is lower than that of the

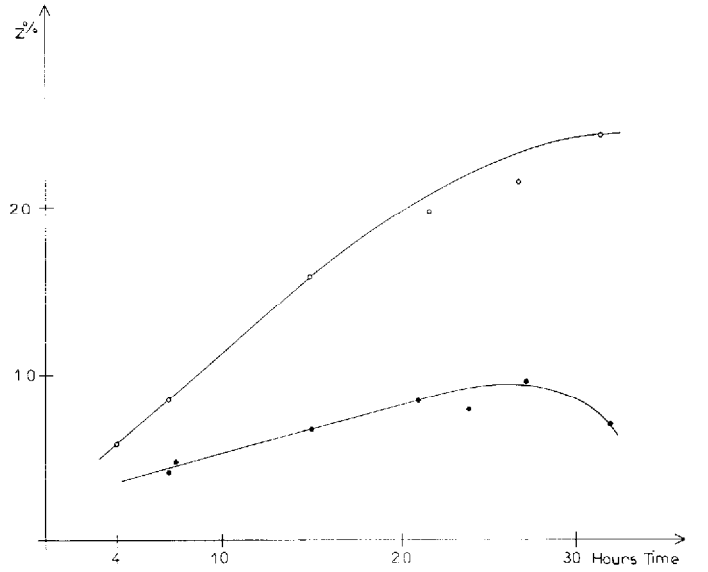


Fig. 4. Coupling to epoxy-activated Sepharose 6B *versus* reaction time for *ortho*-hydroxy (○) and *meta*-hydroxycinnamic acid (●).

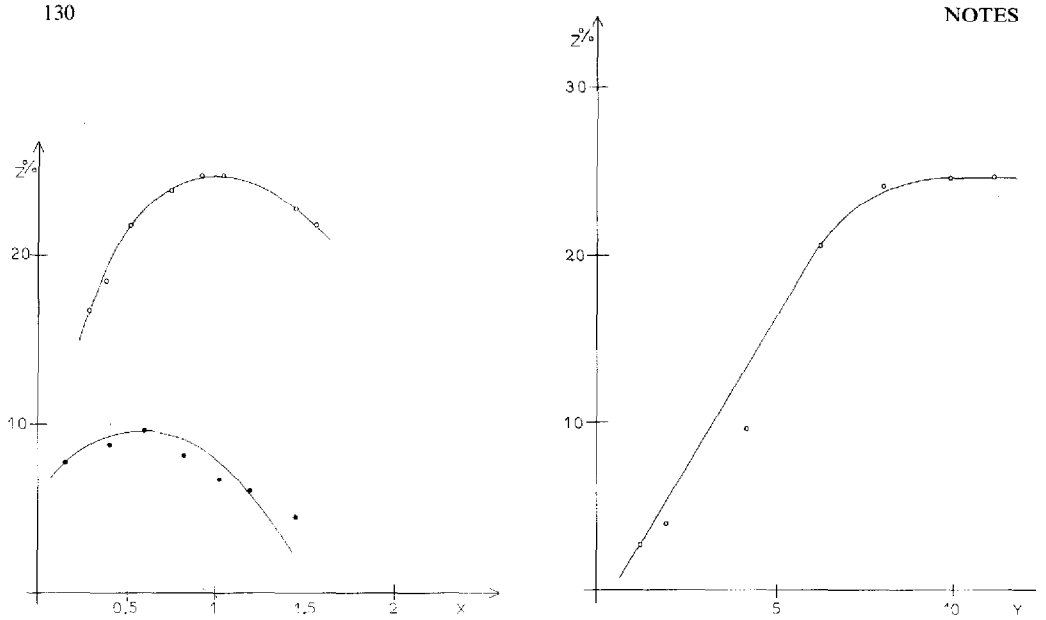


Fig. 5. Coupling to epoxy-activated Sepharose 6B *versus* sodium hydroxide concentration for *ortho*-(○) and *meta*-hydroxycinnamic acid (●).

Fig. 6. Coupling to epoxy-activated Sepharose 6B *versus* the concentration of *ortho*-hydroxycinnamic acid.

ortho isomer. Consequently, the hydroxide-ion concentration is higher in the case of the *meta* isomer case than in that of the *ortho* isomer. The difference in the observed reactions is thus explained by the competition between epoxide hydrolysis and phenol ether formation.

Optimization of sodium hydroxide concentration. The influence of sodium hydroxide concentration on the yield was studied at 30°C using aliquots of 500 mg gel and with $Y = 10$. Fig. 5 shows Z *versus* the sodium hydroxide concentration, which participates in the phenoxide–phenol equilibrium. In the case of the *ortho* isomer, the optimum yield is obtained when $X = 1$. Initially, due to the increase in the phenate concentration, the coupling yield increases and reaches a maximum. At $X > 1$, the sodium hydroxide is in large excess and consumes the epoxy rings so quickly that hydroxycinnamate coupling no longer increases. The behaviour of the *meta* isomer is similar, but the maximum yield is attained at $X = 0.6$. This difference is explained by the hydroxide-ion concentration, which is higher in the case of the *meta* isomer, and by the higher reactivity of the *ortho* isomer.

Optimum hydroxycinnamic acid concentration. The coupling reaction depends on the hydroxycinnamic acid concentration (Fig. 6). A series of reactions was carried out with $X = 1$, at 30°C for 32 h. For the *ortho* isomer, when $Y = 8$, the yield no longer increases. The hydrolysis of the epoxy ring can be decreased by increasing the hydroxycinnamic acid concentration. However, at the same time, the hydroxide-ion concentration increases and a yield of 24% cannot be exceeded.

Effect of the temperature. The reaction yield increases with temperature. In order to avoid gel degradation, the temperature was maintained below 60°C. Fig. 7 shows the amount of hydroxycinnamic acid coupled to the gel as a function of temperature.

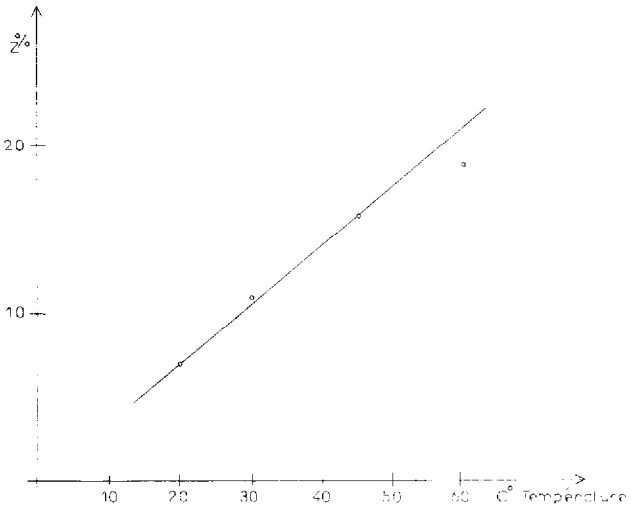


Fig. 7. Coupling to epoxy-activated Sepharose 6B *versus* temperature for *ortho*-hydroxycinnamic acid.

CONCLUSION

In this work it has been shown that the addition of *ortho*- and *meta*-hydroxycinnamic acids to epoxy rings in a basic medium leads to the formation of a phenol ether. The four studied parameters, sodium hydroxide and ligand concentrations, time and temperature, allow optimization of the yield and control of the percentage of hydroxycinnamic acid grafted onto the agarose gel.

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