

Short Communications

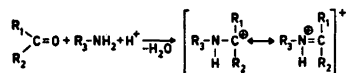
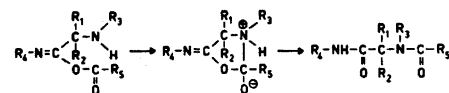
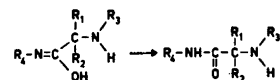
The Use of Isocyanides for the Attachment of Biologically Active Substances to Polymers

R. AXÉN, P. VRETLAD and J. PORATH

Group of Applied Biochemistry in Uppsala
(Swedish Board for Technical Development),
Institute of Biochemistry, Box 531,
S-751 21 Uppsala 1, Sweden

Biospecific adsorbents^{1,2} and insolubilized enzymes³ are receiving increasing attention as tools for "affinity chromatography" and catalytic bed reactions. A great number of methods are used to couple biologically active substances to polymers. Most of them are based on the reactivity of amino groups. We like to report a method with great inherent possibilities since it may form the desired, stable linkage also by means of reactions with other groups. The method is based on the following reactions:

I. Formation of Immonium ions.

II. α -Addition to isocyanides ($R_4-N=C$) by immonium ions and carboxylic ions (R_5COO^-) and rearrangement of the adducts (Ugi reaction).III. α -Addition to isocyanides ($R_4-N=C$) by immonium ions and hydroxylic ions and rearrangement.

The reactions involved in the above scheme have particularly been studied by Ugi and his collaborators in homogeneous medium,⁴ but not, to our knowledge, for

cases where one or more of the functional groups R_1 — R_5 is contributed by a polymer. The formation of an immonium ion structure from an aldehyde or ketone and an amine (I) is a characteristic feature of the method. The immonium ion forms a highly reactive intermediate with an isocyanide. The intermediate is very susceptible to addition reactions with such nucleophilic agents as carboxylic (II) or hydroxylic ions (III). A stable amide is finally formed by an intramolecular rearrangement (II and III).

In practice, the coupling of a chemical substance to a polymer carrier is often simple although the reaction mechanism is usually complicated. The substances to be attached to the carrier polymer may contain amino, carboxyl, keto, aldehyde, or isocyanide groups. The reaction occurs in aqueous solution under conditions mild enough to allow application to enzymes and other proteins.

Among the polymers that we have used as matrix partners for the production of insolubilized enzymes or specific adsorbents the following may be mentioned. Sephadex and agarose substituted with carboxyl groups; polyacrylamide and agarose substituted with amino groups; carbonyl derivatives of agarose; and epichlorohydrin-cross-linked agarose, keratin and wool.

In addition to proteins, blood group substances, peptides, amino acids, biotin, estrone, and cortisone have been coupled to insoluble polymers by the isocyanide method. Soluble polymers have also been used in the reaction: insulin was coupled to albumin and soluble keto-dextran. More detailed descriptions of these latter products will be published elsewhere. The procedures of fixation will be illustrated here for the case of chymotrypsin (Table 1).

The coupling of chymotrypsin to carriers is quite efficient when applied to carboxyl and amino polymers having a high degree of substitution. The catalytic activities are low, however. Very likely the isocyanide reaction involves both intra- and intermolecular protein reactions. The high

Table 1. Chymotrypsin conjugates of a series of carrier polymers. The conjugates are synthesized by means of 3-dimethylaminopropyl isocyanide. The complete reaction mixture must contain amino-, carboxyl-, carbonyl- and isocyanide functions. Activity determinations of the synthesized catalysts toward a small-molecular substrate (ATEE) and a large-molecular substrate (casein). Activity ratios (bound to free CHY) are calculated.

Carrier (50 mg)	Reaction conditions: mg CHY + μ l isocyanide + additional reagent if any (μ l); reaction time in h	Amount of fixed CHY in mg/g conjugate	Activity toward ATEE		Activity ratio toward casein %
			pH-opt.	Activity ratio %	
CM-Sephadex C-50	5 + 25 + acetaldehyde (25); 1	55	10.0	15	3
»	5 + 125 + » (125); 1	70	10.0	5	—
»	50 + 125 + » (125); 1	395	10.0	5	—
»	35 + 25; 1	2	—	—	—
CM-agarose	35 + 25 + acetaldehyde (25); 1	60	9.8	10	5
Lysine-Sepharose	20 + 25 + » (25); 6	15	9.2	50	20
Cross-linked aminoethyl polyacrylamide	20 + 60 + » (60); 3	45	10.0	10	5
Enzaoryl AA	3 + 25 + » (25); 6	48	9.4	30	3
»	35 + 25 + » (25); 6	135	9.5	25	2
Periodate oxidized Sepharose	12 + 25; 6	12	9.3	75	40
Periodate oxidized cross- linked Sepharose	15 + 50; 6	70	9.7	40	15
Dimethylsulfoxide (+P ₂ O ₅) oxidized cross-linked Sepharose	20 + 25; 6	55	9.3	40	10
Dimethylsulfoxide (+Ac ₂ O) oxidized cross-linked Sepharose	35 + 25; 6	40	9.7	50	20
Keratin	35 + 25 + acetaldehyde (25); 6	—	9.5	10 μ mol min ⁻¹ mg ⁻¹ conjugate	—

activity of chymotrypsin fixed to lysine-agarose indicates that this disadvantage can be overcome. The structure of the carrier as well as the degree of protein substitution are important factors for the activity of the insolubilized enzyme.

The fixation of chymotrypsin to carbonyl polymers leads to conjugates in which the fixed protein is very active. The low coupling yields are due to the low carbonyl content of the polymers used. We are presently developing new carbonyl containing polymers.

Chymotrypsin can be attached to keratin and wool with retention of activity. We have also prepared Sepharose carrying both papain and chymotrypsin. A papain-conjugate of Sepharose was prepared by the cyanogen bromide method.⁵ In a second step chymotrypsin was conjugated onto the papain-moiety of the papain-Sepharose by the isocyanide procedure. The papain

activity of "the double enzyme" toward *N*-benzoyl-L-arginine ethyl ester was 80 % of the activity of the free enzyme while the chymotrypsin activity toward *N*-acetyl-L-tyrosine ethyl ester was only 5 %. The pH-optimum of the double enzyme toward *N*-acetyl-L-tyrosine ethyl ester was 9.4, and the optimum toward *N*-benzoyl-L-arginine ethyl ester was 6.8. When both substrates were incubated simultaneously the pH-optimum was 8.0.

Experimental. 3-Dimethylaminopropyl isocyanide was synthesized from the corresponding amine by formylation with ethyl formate and subsequent elimination of water by phosgene according to Ugi *et al.*⁴

Cross-linking of Sepharose with epichlorohydrin. Sepharose[®] was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Sepharose 6B was cross-linked with epichlorohydrin according to Porath and Eklund.⁷

in the following way: 10 ml wet Sepharose was treated with 8 ml 1 N sodium hydroxide and 2 ml epichlorohydrin at 60° for 20 h under stirring. The reaction product was washed with 1 N sodium hydroxide and water.

Carboxyl polymers. Sephadex® C-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) and carboxymethyl-agarose were used as solid supports. Carboxymethyl-agarose was prepared essentially according to Schell.⁸

Amino polymers. Enzaeryl® AA purchased from Koch-Light Laboratories, Colnbrook, Buckinghamshire, England was applied as an amino-group-containing support. Cross-linked polyacrylamide (P200) from Bio-Rad Laboratories, Richmond, California, was converted to a derivative with ethylenediamine according to Inman and Dintzis.⁹

Agarose-lysine ethyl ester containing free amino groups was prepared by cyanogen bromide activation at pH 11 of Sepharose 2 B (2 g wet gel) and subsequent coupling of lysine ethyl ester (20 mg) at pH 9.¹⁰

Keratin was purchased from Nutritional Biochemical Corp., Cleveland, Ohio, USA.

Carbonyl polymers. Introduction of carbonyl groups into Sepharose or epichlorohydrin cross-linked Sepharose was achieved by (A) periodate oxidation¹¹ or (B and C) by dimethyl sulfoxide oxidation.^{12,13}

A. Wet Sepharose 4 B (2.5 g) was dispersed in 5 ml 0.1 M sodium metaperiodate and the reaction allowed to proceed for 2 h at 4° in the dark. The gel was washed with 0.1 M sodium bicarbonate and 1 mM hydrochloric acid and was immediately used for coupling.

Epichlorohydrin-cross-linked Sepharose was similarly treated with 0.5 M sodium metaperiodate for 20 h at room temperature.

B. Cross-linked Sepharose (1 g), 12 ml dimethyl sulfoxide, and 5 ml acetic anhydride were mixed and allowed to react at 40° for 90 min under stirring. The gel was washed on a glass filter with acetone and thereafter dispersed in 15 ml 0.4 M hydrochloric acid and stirred gently at 35° for 2 h. The gel was finally washed with water.

C. Cross-linked Sepharose (0.5 g), 0.9 g phosphorus pentoxide, and 0.4 g dimethyl sulfoxide were allowed to react in 25 ml dimethyl formamide for 2 h at 65°. The reaction mixture was poured into ice-water and the product was washed carefully with water.

Chemical fixation of chymotrypsin. To 50 mg-portions of carrier polymer suspended in 2 ml of distilled water different amounts of chymotrypsin were added (Table 1). 3-Dimethylaminopropyl isocyanide was added, and in the cases of amino- and/or carboxyl-group containing polymers acetaldehyde was

also included. The pH was adjusted to 6.5 and kept constant by addition of 0.5 M hydrochloric acid in a pH-stat. After completion of the reaction (normally 6 h) the excess of reagents was removed by washing on a glass filter. The product was then washed in a small column combined with a peristaltic pump. The following solutions were used in the order indicated: 0.1 M sodium borate buffer 1 M in sodium chloride of pH 8.5 (48 h), 0.1 M acetate buffer 1 M in sodium chloride of pH 4.1 (24 h), and finally 0.01 M acetate buffer of pH 4.1 (24 h). The products were stored in the acetate buffer at 4°. The amount of fixed enzyme was determined by amino acid analysis after acid hydrolysis.¹⁴ Chymotrypsin and fixed chymotrypsin were assayed titrimetrically at 23° toward 2 ml of a substrate solution 0.01 M in *N*-acetyl-L-tyrosine ethyl ester (ATEE), 0.05 M in potassium chloride and 5 % in ethanol.⁵

The caseinolytic determinations were done according to Bergmeyer¹⁵ as modified earlier for the assay of insoluble conjugates.⁵

The apparent pH-optima of the conjugates were determined. Appreciable shifts in pH-optimum were recorded in the case of ester substrates, but practically no shifts were observed with casein as substrate. The activities of the fixed enzyme were compared with the activities of the free enzyme. The activity ratio in % was calculated (Table 1).

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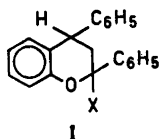
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The NMR Spectra of Some Chroman Derivatives

GUST-AD. HOLMBERG and
RAINER SJÖHOLM

*Institutionen för organisk kemi, Åbo
Akademi, SF-20500 Åbo 50, Finland*

Some years ago, 2,4-diphenyl-2-methylchroman (I a) was synthesised in this laboratory and two isomers melting at 118–119° and 83.0–83.5° were isolated.¹ Because the molecule contains two asymmetric carbon atoms, it is evident that the isolated isomers were racemic mixtures in which the enantiomers of one mixture were diastereomers of those of the other mixture. The configurations of the substances were not established at that time. The NMR spectra of the substances, however, make it possible to determine these configurations.



- X = (a) CH₃
(b) C₆H₅
(c) OC₂H₅

In order to obtain more information, the NMR spectra of 2,2,4-triphenylchroman (I b) and 2,4-diphenyl-2-ethoxychroman (I c) were taken in addition to those of the two 2,4-diphenyl-2-methylchroman isomers. In all cases, the three non-aromatic hydrogen atoms formed an ABX system from which the chemical shifts of these atoms could be derived.² The results are collected in Table 1, which also contains

information on chemical shifts of the other protons excluding the aromatic protons. It is evident from the chemical shifts that the A and B protons are the hydrogen atoms at position 3 and the X protons the hydrogen atoms at position 4. Important features of the spectra are the small difference between the shifts of the A and B protons of the low-melting 2,4-diphenyl-2-methylchroman and the positions of the X proton shifts. An interpretation of the spectra is most easily achieved by an analysis of the possible conformations.

A chromane derivative most likely exists in a half-chair or a half-boat conformation. In Fig. 1, the half-chair con-

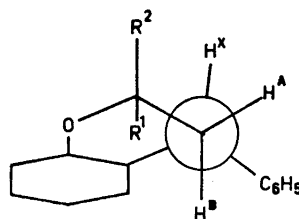


Fig. 1. Half-chair conformation of chromane derivative projected on a plane perpendicular to the bond between C(3) and C(4).

formation is projected on a plane perpendicular to the bond between the carbon atoms in positions 3 and 4. If the phenyl group in position 4 occupies the semi-equatorial position, the coupling constants J_{BX} and J_{AX} should be of the order of 10–15 and 5 Hz, respectively, according to the Karplus rule. If the phenyl group is in the semi-axial position, the two coupling constants should be almost equal and of the order of about 5 Hz. A similar discussion of the half-boat conformation leads to almost identical results. Because the experimentally determined coupling constants are about 13 and 5 Hz, the phenyl group in position 4 occupies the semi-equatorial position.

If the pyran rings exist in the half-chair conformation and a phenyl group in position 2 is in the *trans* position relative to the phenyl group in position 4, the former phenyl group should be in the axial position (R^2 in Fig. 1). An examination of Stuart scale models of such molecules shows that the phenyl group in position 2 turns its positively shielding sector³ towards the X proton. A phenyl group in the *cis* position