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Liquid-Phase Peptide Synthesis by Fragment Condensation on Soluble Polymer Support. 7.[1,2] Influence of Polymer Support Cross-Linking on the Coupling Reactions of Large Carboxyl-Component Peptides with Polymer-Bound Amino-Component Peptides

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ABSTRACT: The influence of polymer support cross-linking on the relative coupling efficiencies of carboxyl-component peptides having various chain lengths was investigated in their coupling reactions with terminal free amino groups of an amino acid or peptide anchored to a polymer support. The relative coupling efficiencies of Boc-$(Leu_3Pro_2Gly)_n$-OH (n = 1, 2, and 4) and Boc-Ala-OH were determined by their competitive coupling reactions with H-Phe-polymer (soluble polystyrene) (**1**), H-Leu_3Phe-resin [copoly(styrene–1% divinylbenzene)] (**2**), or H-Leu_9Phe-resin [copoly(styrene–1% divinylbenzene)] (**3**) in dimethylformamide (DMF) using dicyclohexylcarbodiimide (DCC) and 1-hydroxy-1*H*-benzotriazole (HOBt) as coupling reagents. In the competitive reactions of the Boc-oligopeptides and Boc-Ala-OH with soluble polymer **1**, the relative coupling efficiencies decreased very little with increasing peptide chain length of the Boc-oligopeptides. On the other hand, in the competitive reactions with cross-linked resins **2** and **3**, the relative coupling efficiencies of the Boc-oligopeptides decreased with increasing peptide chain length, indicating restricted permeability of large peptides into cross-linked resin matrices **2** and **3**. All of the relative coupling efficiencies of the Boc-oligopeptides in the competitive reactions with cross-linked resin **3** were found to be lower than those of the Boc-oligopeptides in the reactions with cross-linked resin **2**, indicating that the permeability of the Boc-oligopeptides was further restricted by additional cross-linking caused by a β-sheet formation of the H-Leu_9Phe sequence on resin **3**. The total coupling yields in the competitive coupling reactions of the Boc-oligopeptides and Boc-Ala-OH with resin **3** are lower than those in the reactions with soluble polymer **1** and cross-linked resin **2**.

Peptide synthesis by fragment condensation on polymer supports has been expected to be one of the most promising methods for syntheses of large peptides and proteins.[1] However, one serious problem is low yields in some fragment condensation reactions on cross-linked resin supports.[3] The decrease in coupling yields is believed to result from the restricted permeability of carboxyl-component peptides into resin matrices. In the previous paper,[1] the influence of the peptide chain length of Boc-oligopeptides on the coupling efficiencies of their C-terminal amino acids was investigated in their coupling reactions with terminal free amino groups of amino acids anchored to polymer supports. In the coupling reactions with amino components anchored to soluble polystyrene and to copoly(styrene–1% divinylbenzene), little influence of the peptide chain length was observed with Boc-oligopeptides consisting of 3–10 amino acid residues. On the other hand, in the reactions with amino components anchored to copoly(styrene–2% divinylbenzene), the increase in the peptide chain lengths of Boc-oligopeptides was found to reduce remarkably the coupling efficiencies of their C-terminal amino acids. In the reactions, it was also shown that there were terminal free amino groups which were sterically inaccessible for Boc-oligopeptides consisting of 5–10 amino acid residues.

In addition, in our recent IR study on the conformation of H-Leu_nPhe-resins (n = 3, 6, 9, and 15) in various organic solvents,[4] it was made clear that peptide chains anchored to copoly(styrene–1% divinylbenzene) easily interacted with each other through hydrogen bonding even at the low loading of peptide chains: about 1 mol % per styrene monomeric unit. This result indicates that the resin support is sufficiently flexible for the pendant peptide chains' interactions. The flexible property of copoly(styrene–1% divinylbenzene) has also been reported recently in a few papers.[5,6] The onset of a β-sheet structure by hydrogen bonding between pendant peptide chains brings about additional cross-linking of the polymer network. Thus, in that case, the coupling efficiencies of carboxyl-component peptides are expected to be depressed with increasing peptide chain length. On the other hand, as long as both the carboxyl- and amino-component peptides have

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Table I
Relative Coupling Efficiencies and Total Coupling Yields

amino-component peptide polymer	Boc-peptide n	content of amino acid in resulting peptide polymer, μmol/g					content of peptide, μmol/g	rel coupling efficiency[a]	total coupling yield, %	recov of Phe,[b] %
		Pro	Gly	Leu	Ala	Phe				
1	1	74.6	39.9	113.6	36.2	85.9	38.0[c]	1.05	90[d]	104
1	2	140.0	75.0	214.4	38.5	77.9	35.8[c]	0.93	95	97
1	4	260.6	131.8	405.0	35.2	74.1	33.2[c]	0.94	93	96
2	1	102.7	49.8	438.6	47.3	108.0	50.8[e]	1.08	91	93
2	2	170.5	80.4	508.0	48.9	102.5	41.8[e]	0.85	89	91
2	4	242.8	115.9	642.8	60.8	109.2	29.9[e]	0.49	88	98
3	1	25.4	17.6	594.0	28.3	58.8	17.6[f]	0.62	78	81
3	2	40.1	24.1	607.5	34.9	58.5	12.1[f]	0.34	81	81
3	4	25.4	18.3	570.4	39.0	59.6	4.6[f]	0.12	74	82

[a] (Peptide content)/(Ala content). [b] Based on the content of Phe calculated from the coupling yield. [c] {(Pro content) + (Gly content) + (Leu content)}/6n. [d] Value calculated from the Phe content justified as the recovery of Phe to be 100%. [e] {(Pro content) + (Gly content)}/3n. [f] (Gly content)/n.

a randomly coiled structure in their reactions using soluble polystyrene as a support, it is expected that the peptide chain lengths of carboxyl-component peptides have little or no influence on their coupling efficiencies.

In this paper, we first investigate the influence of the peptide chain lengths of carboxyl-component peptides consisting of 6–24 amino acid residues on their coupling efficiencies in their reactions with amino components anchored to soluble polystyrene. The influence both of polymer support cross-linking and of secondary structures of the amino-component peptides anchored to copoly(styrene–1% divinylbenzene) on the coupling efficiencies is next investigated using the resin-bound amino-component peptides having a randomly coiled structure and a partially developed β-sheet structure, respectively. Here, the sequential polypeptides Boc-$(Leu_3Pro_2Gly)_n$-OH (n = 1, 2, and 4) are used as carboxyl-component peptides, which have been examined to have predominantly a randomly coiled structure under the "peptide segment separation" concept as described previously.[7-10] The relative coupling efficiencies of the Boc-oligopeptides and Boc-Ala-OH are determined by their competitive coupling reactions with terminal free amino groups of amino acids or peptides anchored to the soluble polymer and cross-linked resin supports.

Experimental Section

Materials and IR Measurements. Soluble H-Phe-*p*-(oxymethyl)phenylacetamidomethylated polystyrene (1) (Phe content 83.5 μmol/g) was prepared according to the method described previously.[11,12] Copoly(styrene–1% divinylbenzene) beads of 200–400 mesh, Bio-Beads S-X1, were purchased from Bio-Rad Laboratories. Cross-linked resin-bound oligoleucines, H-Leu_3Phe-resin (2) (Phe content 122 μmol/g) and H-Leu_9Phe-resin (3) (Phe content 74 μmol/g) were those prepared in the previous paper,[13] in which step-by-step coupling of Boc-Leu_3-OH was performed on cross-linked H-Phe-*p*-(oxymethyl)phenylacetamidomethylated polystyrene using dicyclohexylcarbodiimide (DCC) and 1-hydroxy-1*H*-benzotriazole (HOBt) as coupling reagents. The Leu:Phe ratios of the peptide resins 2 and 3 had been determined to be 3.0 and 8.9, respectively, by amino acid analyses of acid hydrolysates of the peptide resins.[13] Boc-$(Leu_3Pro_2Gly)_n$-OH (n = 1, 2, and 4) were those prepared previously.[8] The IR absorption spectra of the resin 3 in tetrahydrofuran and in dimethylformamide (DMF) were measured by means of a Jasco Model DS-701G spectrometer. Experimental details of IR measurements were reported previously.[4]

Relative Coupling Efficiencies of Boc-Oligopeptides and Boc-Ala-OH in the Competitive Coupling Reactions (General Method). H-Phe-polymer (1), H-Leu_3-Phe-resin (2), or H-Leu_9-Phe-resin (3) (100 mg) was dissolved or swollen in DMF (5 mL), and 5 equiv of Boc-$(Leu_3Pro_2Gly)_n$-OH (n = 1, 2, or 4) and 5 equiv of Boc-Ala-OH were added into the solution or the suspension. To the reaction mixture cooled to 0 °C were added 12 equiv of HOBt and 10 equiv of DCC. The mixture was stirred at 0 °C for 2 h and at room temperature for 2 days and then poured into water (50 mL). The polymer precipitated was filtered off, washed thoroughly with ethanol, and dried in vacuo at 50 °C. The peptide polymers thus obtained were subjected to acid hydrolysis with propionic acid–12 M HCl (2:1 (v/v)) for 72 h at 120 °C in evacuated, sealed tubes, followed by amino acid analysis. In our preliminary experiments, recoveries of Pro, Gly, Leu, and Phe of Boc-amino acids bound to soluble polystyrene were found to have relative values of 0.94, 1.06, 1.01, and 1.00, respectively, after acid hydrolysis with propionic acid–12 M HCl (2:1 (v/v)) for 72 h at 120 °C compared to the recoveries after acid hydrolysis with the same reagents for 24 h at the same temperature. In our previous studies, fairly good recoveries of Pro and Leu of Boc-peptides bound to cross-linked polystyrene have been also obtained after acid hydrolysis with the same reagents for 6–8 days at 120 °C.[14] Amino acid analysis was carried out with a Shimadzu HPLC LC-3A all amino acid analysis system. Relative coupling efficiencies of Boc-$(Leu_3Pro_2Gly)_n$-OH (n = 1, 2, and 4) were obtained from the ratios of the peptide contents to the Ala content as shown in Table I.

Results and Discussion

For the purpose of elucidating the influence of the peptide chain lengths of Boc-$(Leu_3Pro_2Gly)_n$-OH (n = 1, 2, and 4) on the coupling efficiencies in their reactions with the soluble H-Phe-polymer (1), the relative coupling efficiencies of Boc-$(Leu_3Pro_2Gly)_n$-OH (n = 1, 2, and 4) and Boc-Ala-OH were determined by competitive coupling reactions of the Boc-oligopeptide and Boc-Ala-OH with the soluble H-Phe-polymer (1). The relative coupling efficiencies of the Boc-oligopeptides were plotted against the logarithm of the molecular weights of the Boc-oligopeptides (Figure 1). These results show that, using the soluble H-Phe-polymer (1) as an amino component, the decrease in the relative coupling efficiencies is very small with increasing peptide chain length of Boc-$(Leu_3Pro_2Gly)_n$-OH (n = 1, 2, and 4). These peptides have been shown to have predominantly a randomly coiled structure in highly polar solvents by CD, molar rotation, and NMR studies.[10,15,16] In particular, the NMR studies on Boc-$(Leu_3Pro_2Gly)_n$-OBzl (n = 1–4) in dimethyl-d_6 sulfoxide strongly suggest the following conformational features: (1) The local conformations of C-terminal Pro_2Gly segments of all the peptides are similar to each other regardless of the chain lengths of the sequential polypeptides. (2) The internal $Pro_2GlyLeu_3$ segments of Boc-$(Leu_3Pro_2Gly)_n$-OBzl (n = 2–4) all have the same conformation. A randomly coiled structure of Boc-$(Leu_3Pro_2Gly)_n$-OBzl (n = 1–4) regardless of the peptide chain length was attributed to "peptide segment separation" via the insertion of tertiary peptide bonds at suitable intervals.[7-10] Thus, the peptide sequence

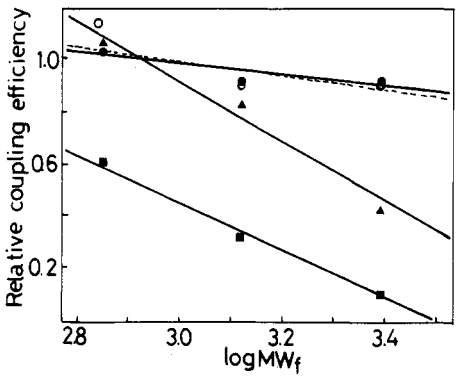

Figure 1. Influence of the peptide chain lengths of Boc-$(Leu_3Pro_2Gly)_n$-OH (n = 1, 2, and 4) on their coupling efficiencies in the competitive coupling reactions of Boc-$(Leu_3Pro_2Gly)_n$-OH (n = 1, 2, and 4) and Boc-Ala-OH with soluble polymer 1 and cross-linked resins 2 and 3. (●) H-Phe-polymer (1); ▲ H-Leu_3Phe-resin (2); (■) H-Leu_9Phe-resin (3). The dashed line (○) indicates the result obtained with H-Leu_9Phe bound to soluble polystyrene support in N-methylpyrrolidone.

$-(Leu_3Pro_2Gly)_n-$ is expected to be subjected to sufficient solvation in DMF, which is used as solvent in the competitive coupling reactions, and have a randomly coiled structure after Boc-$(Leu_3Pro_2Gly)_n$-OH (n = 1, 2, and 4) is coupled to soluble H-Phe-polymer (1). Therefore, the small decrease in the relative coupling efficiencies of the Boc-oligopeptides in their reactions with H-Phe-polymer (1) is attributed to little influence of the peptide chain length up to 24 amino acid residues on the coupling efficiencies of the Boc-oligopeptides. Similar results have been obtained in the competitive reactions of the Boc-oligopeptides and Boc-Ala-OH with H-Leu_9Phe bound to soluble polystyrene in N-methylpyrrolidone as shown in Figure 1, although the H-Leu_9Phe-polymer (Phe content 82 μmol/g) has been detected to have partially a β-sheet structure in the solvent by IR.[17] It has been reported that the reactivity of the terminal amino group is significantly reduced in poly(oxyethylene)-bound oligomers exhibiting β-structures compared to poly(oxyethylene)-bound oligomers in α-helical or randomly coiled conformations in the peptide synthesis by stepwise elongation.[18-20] However, the results mentioned above indicate that a partial contribution of a β-sheet structure to the conformation of a peptide bound to a soluble polystyrene support has little or no influence on the coupling efficiencies of the Boc-oligopeptides. The influence of secondary structures of peptides bound to a soluble polystyrene support on the relative coupling efficiencies of Boc-oligopeptides in a randomly coiled structure will be investigated in the next paper.[17]

Here, in order to investigate the influence both of polymer support cross-linking and of secondary structures of resin-bound amino-component peptides bound to cross-linked polystyrene support on the coupling efficiencies, the conformations of H-Leu_3Phe-resin (2) and H-Leu_9Phe-resin (3) were examined by IR spectroscopy in a swollen state. The conformation of H-Leu_3Phe-resin (2) in the swollen state has been shown to have a randomly coiled structure in various polar solvents such as methylene chloride, tetrahydrofuran, and hexamethylphosphorotriamide (HMPA).[4,13] The IR spectra in the amide I region of H-Leu_9Phe-resin (3) in several solvents having various polarities are assembled in Figure 2 from our previous paper.[4] The bands at 1628 cm^{-1} in the spectra of resin 3 in carbon tetrachloride, methylene chloride, and tetrahydrofuran indicate that H-Leu_9Phe sequences, anchored to copoly(styrene–1% divinylbenzene) can easily interact

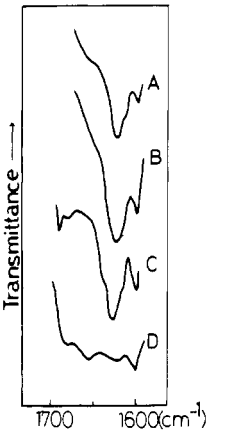

Figure 2. IR spectra of the H-Leu_9Phe-resin (3) in the amide I region in a swollen state: (A) in carbon tetrachloride; (B) in methylene chloride; (C) in tetrahydrofuran; (D) in HMPA. The absorption band at 1603 cm^{-1} is due to aromatic rings of the polystyrene support.

with each other through intermolecular hydrogen bonding as pointed out previously.[4] The band centered at 1627 cm^{-1} in the spectrum of resin 3 in HMPA also indicates that the H-Leu_9Phe sequence still has partially a β-sheet structure in HMPA, although a partial β → α and/or coiled conformational transformation takes place in HMPA. A broad band centered at 3280 cm^{-1} was observed in the spectrum of H-Leu_9Phe-resin (3) in DMF, which indicates that the H-Leu_9Phe sequence has partially a β-sheet structure in DMF, although the spectrum was rather complicated with bands of the solvent and is not shown here. Thus, the conformations of H-Leu_3Phe-resin (2) and H-Leu_9Phe-resin (3) in DMF are randomly coiled and partially developed β-sheet structures, respectively.

Here, we can make clear the influence both of polymer support cross-linking and of secondary structures of amino-component peptides anchored to copoly(styrene–1% divinylbenzene) on the coupling efficiencies. Figure 1 also shows all the relative coupling efficiencies of the Boc-oligopeptides in the coupling reactions with the amino-component peptides anchored to the cross-linked resin supports 2 and 3. The results indicate that, except the value in the reaction of Boc-Leu_3Pro_2Gly-OH with resin 2, they are lower than those of Boc-$(Leu_3Pro_2Gly)_n$-OH (n = 1, 2, and 4) in their coupling reactions with the soluble polymer 1. It is also observed that in the reactions with resins 2 and 3, the relative coupling efficiencies of the Boc-oligopeptides decrease with increasing peptide chain length. These results exactly show the restricted permeability of carboxyl-component peptides into the resin matrices. All the relative coupling efficiencies of the Boc-oligopeptides in the reactions with resin 3 are lower than those of the Boc-oligopeptides in the reactions with resin 2, indicating that additional cross-linking caused by a β-sheet formation of the H-Leu_9Phe sequence on resin 3 further restricts the permeability of the Boc-oligopeptides into the resin matrix. The linear relationship between the relative coupling efficiencies of the Boc-oligopeptides in the reactions with resins 2 and 3 and the logarithm of their molecular weights also indicates that the diffusion of the carboxyl-component peptides into resin matrices 2 and 3 is controlled analogously as well as that in the gel permeation chromatography.

The total coupling yields of the Boc-oligopeptides and Boc-Ala-OH in the competitive coupling reactions are summarized in Table I. The total coupling yields in the reactions with cross-linked resin 3 are lower than those in the reactions with soluble polymer 1 and cross-linked resin

2. These results indicate that the terminal free amino groups which are inaccessible even for the Boc-Ala-OH are present in resin **3**.

Conclusion

In the competitive reactions of Boc-$(Leu_3Pro_2Gly)_n$-OH (n = 1, 2, and 4) and Boc-Ala-OH with the soluble polymer (**1**), very little decrease in the relative coupling efficiencies of the Boc-oligopeptides with increasing peptide chain length is observed as shown in Figure 1. In contrast with these observations, in the competitive reactions with cross-linked resins **2** and **3**, their relative coupling efficiencies decreased remarkably with increasing peptide chain length. These results indicate that peptide syntheses by fragment condensation on soluble polymer supports are performed with little influence of the peptide chain length of the Boc-oligopeptides consisting of up to 24 amino acid residues on the yields of coupling reactions, as long as both the carboxyl-component peptides and polymer-bound amino-component peptides have a randomly coiled structure. The "peptide segment separation" concept is demonstrated to be very useful for keeping peptides as randomly coiled structures and to be applicable for any peptide having arbitrary amino acid sequences by the insertion of temporary protecting groups into X–Y peptide bonds.[7-10] Thus, the combination of both peptide syntheses by fragment condensation on soluble polymer supports and the "peptide segment separation" concept has promising versatility for syntheses of pure large peptides and proteins, giving high coupling yields and easy processing procedures.

Registry No. Boc-(Leu_3Pro_5Gly)-OH, 91649-94-4; Boc-$(Leu_3Pro_2Gly)_2$-OH, 91649-95-5; Boc-$(Leu_3Pro_2Gly)_4$-OH, 91649-96-6; Boc-Ala-OH, 15761-38-3.

References and Notes

(1) For part 6 in this series, see S. Isokawa, N. Kobayashi, R. Nagano, and M. Narita, *Makromol. Chem.*, **185**, 2065 (1984).
(2) The abbreviations for amino acids are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1972). The amino acid symbols except Gly denote the L configuration. Additional abbreviations: Boc, *tert*-butoxycarbonyl; Bzl, benzyl.
(3) H. Yajima and Y. Kiso, *Chem. Pharm. Bull.*, **22**, 1087 (1974).
(4) M. Narita, Y. Tomotake, S. Isokawa, T. Matsuzawa, and T. Miyauchi, *Macromolecules*, **17**, 1903 (1984).
(5) B. Kim, P. Kirszensztejn, D. Bolikal, and S. L. Regen, *J. Am. Chem. Soc.*, **105**, 1567 (1983).
(6) K. K. Bhargava, V. K. Sarin, N. L. Trang, A. Cerami, and R. B. Merrifield, *J. Am. Chem. Soc.*, **105**, 3247 (1983).
(7) M. Narita, T. Fukunaga, A. Wakabayashi, K. Ishikawa, and H. Nakano, *Int. J. Pept. Protein Res.*, **23**, 306 (1984).
(8) M. Narita, S. Nagasawa, J. Y. Chen, H. Sato, and Y. Tanaka, *Makromol. Chem.*, **185**, 1069 (1984).
(9) M. Narita, K. Ishikawa, H. Nakano, and S. Isokawa, *Int. J. Pept. Protein Res.*, **24**, 14 (1984).
(10) M. Narita, N. Ohkawa, S. Nagasawa, and S. Isokawa, *Int. J. Pept. Protein Res.*, **24**, 129 (1984).
(11) A. R. Mitchell, S. B. H. Kent, M. Engelhard, and R. B. Merrifield, *J. Org. Chem.*, **43**, 2845 (1978).
(12) M. Narita, K. Enomoto, H. Ishii, A. Munakata, and S. Isokawa, *Pept. Chem., 1980*, 47 (1981).
(13) M. Narita, S. Isokawa, Y. Tomotake, and S. Nagasawa, *Polym. J.*, **15**, 25 (1983).
(14) S. Isokawa, N. Kobayashi, R. Nagano, and M. Narita, *Makromol. Chem.*, **185**, 2065 (1984).
(15) S. Isokawa, T. Asakura, and M. Narita, *Macromolecules*, in press.
(16) S. Isokawa, I. Tominaga, T. Asakura, and M. Narita, *Macromolecules*, in press.
(17) S. Isokawa, K. Tomita, T. Matsuzawa, and M. Narita, in preparation.
(18) G. M. Bonora, M. Palumbo, and C. Toniolo, *Makromol. Chem.*, **180**, 1293 (1979).
(19) V. N. R. Pillai and M. Mutter, *Acc. Chem. Res.*, **14**, 122 (1981).
(20) S. A. El Rahman, H. Anzinger, and M. Mutter, *Biopolymers*, **19**, 173 (1980).

Polymer-Modified Electrodes. Electrochemical and Photoelectrochemical Polymerization of 1-Vinylpyrene

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ABSTRACT: Polymerization of 1-vinylpyrene in acetonitrile was achieved electrochemically at a conducting SnO_2 electrode by application of anodic potentials and photoelectrochemically at a n-GaAs semiconductor electrode under visible light irradiation. Both polymers exhibited characteristic excimer emission ($\lambda_{max} \sim$ 480 nm) in both films and THF solutions similar to poly(vinylpyrene) synthesized by using Ziegler–Natta catalyst. The lifetime of the excimer emission was ~110 ns. The mechanism of photodegradation of poly(vinylpyrene) was elucidated by using ESR techniques.

Introduction

Polymer-modified electrodes have gained wider attention in recent years because of their importance in electroanalytical and photoelectrochemical applications.[1-5] Electroactive or photoactive groups can either be held electrostatically to polar groups pendent from the nonelectroactive polymer matrix or be attached directly to the backbone of the polymer. In the former type, we have recently reported some interesting features of the electrodes modified by the adsorption of poly(4-vinylpyridine) films containing some anionic dyes.[3-5] Though there have been reports of direct electropolymerization with electroactive groups directly attached to the polymer backbone (e.g. poly(vinylferrocene)[6]), little effort has been directed toward employing photoactive groups in similar polymers. The study of such polymers could reveal significant details

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