Immuno-affinity Purification of Specific Antibodies against Vasoactive Intestinal Polypeptide (VIP) on VIP(1—10)-Linked Polydimethylacrylamide Resin[1)]

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A novel method for immuno-affinity purification of specific antibodies against amino-terminal (N-terminal) porcine vasoactive inestinal polypeptide (p-VIP) was developed. The antiserum VP (No. 6203) elicited by p-VIP–immunoglobulin G (IgG) conjugate was heterogenous and reacted not only with the N-terminal fragments of p-VIP but also with carboxy-terminal (C-terminal) fragments. To obtain specific antibodies against the N-terminal fragment of p-VIP, antiserum VP was purified by column chromatography on N-terminal decapeptide (p-VIP 1—10)-linked polydimethylacrylamide resin. The antibody thus obtained was highly specific to the N-terminal sequence of p-VIP and hardly reacted with the C-terminal fragments of VIP in enzyme-linked immunosorbent assay.

Keywords specific VIP antibody; immuno-affinity purification; p-VIP (1—10)-linked polydimethylacrylamide resin; enzyme immunoassay

Vasoactive intestinal polypeptide (VIP) was first isolated from porcine small intestine by Said and Mutt.[2,3)] This 28-amino acid peptide has various biological effects on mammals, including vasodilation and hypotension,[2,4)] relaxation of non-vascular smooth muscles,[5)] stimulation of pancreatic bicarbonate secretion and bile flow,[6,7)] inhibition of gastric acid secretion,[8)] stimulation of intestinal secretion,[9)] glycogenolysis,[10)] lipolysis and release of insulin and glucagon.[11)] VIP was originally considered to be a gut hormone, but immunohistochemical studies have revealed that VIP has widespread distribution in neurons.[12-14)] VIP-containing neurons are present in the central nervous system including the cerebral arteries, the digestive tract, the genitourinary tract, and the adrenal glands.

At present the structure of porcine,[15,16)] chicken,[17)] bovine,[18)] human,[19)] dog,[20)] guinea pig,[21)] and dogfish[22)] VIPs are known (Table I). We have succeeded in synthesizing porcine,[23)] chicken[24)] and guinea pig[25)] VIPs.

Radioimmunoassay of VIP has been developed by several authors.[26)] However, in terms of safety and specificity, the existing methods are still less than satisfactory. This report describes the preparation of antisera against porcine-VIP (p-VIP) as well as an enzyme-linked immunosorbent assay (ELISA) and new methods for the immunoaffinity purification of the specific antibody from the heterogenous antiserum. We obtained a specific antibody against N-terminal p-VIP by affinity column chromatography on a p-VIP(1—10)-linked polydimethylacrylamide resin,[27)] for further immunological studies.

Experimental

Materials Synthetic p-VIP,[23)] chicken VIP(c-VIP),[24)] guinea pig VIP(GP-VIP),[25)] and Glu[8]-p-VIP,[28)] were used. Synthetic porcine secretin,[29)] human GIP,[30)] and PHI,[31)] were also used. Polydimethylacrylamide resin and Fmoc amino acid were purchased from LKB Pharmacia. Bovine serum albumin (BSA), polyoxyethylenesorbitan (Tween 20), bovine IgG and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co. and alkaline/phosphatase-conjugated goat anti-rabbit IgG was from Capel Laboratories Inc. Other all chemicals were of analytical reagent grade.

Synthesis of Bovine–IgG–VIP Conjugate Bovine–IgG–p-VIP conjugate was synthesized by SH-introduction followed by the 2,4-dinitrophenyl *p*-(2-nitrovinyl)benzoate conjugating method.[32)] A mixture of synthetic p-VIP (5.1 mg) and MTPTT (0.87 mg) in H_2O–THF (20:1) was stirred at room temperature for 4 h and then lyophilized. The resulting powder was treated with 1 M TFMSA–thioanisole/TFA (270 μl) in the presence of *m*-cresol (10 μl) in ice bath for 60 min. Ether was added and the resulting powder was dissolved in H_2O (1 ml). To this solution, an aliquot (100 μl) of a solution of 2,4-dinitrophenyl *p*-(2-nitrovinyl)benzoate (0.96 mg, 2 eq) in THF was added. The mixture was stirred at room temperature for 30 min. The pH of the solution was adjusted to 7.5 with NMM and then bovine IgG (5.29 mg) in H_2O (1 ml) was added. After being stirred for 4 h, the mixture was dialyzed against 2 l of 0.2 N AcOH at 4 °C for 24 h and lyophilized (yield, 9.2 mg).

Preparation of Antisera Antisera against p-VIP were elicited by immunization of rabbits with the above-mentioned bovine–IgG–p-VIP conjugate. Three milligrams of the bovine–IgG–p-VIP conjugate was dissolved in 1.5 ml of saline and emulsified with 1.5 ml of Freund's complete adjuvant and then injected into three rabbits at multiple sites on the back. A second injection was given 14 d later without the mycobacterium (0.5 mg of p-VIP–IgG/rabbit). Booster injections of free p-VIP (2 mg) and the VIP–IgG conjugate (0.5 mg) mixed with alum were repeated several times at 21-d intervals and antisera were harvested bleeding from an ear vein 7 d after the booster injection. The anti-VIP titer was checked. Among three antisera, the high-titer antiserum VP-6203 was obtained.

Determination of Optimal Coating Conditions Assays were performed using 100 μl incubation volumes. The assay buffer was 0.05 M phosphate buffer, pH 7.4, containing 0.5% BSA 250 KIU/ml aprotinin. The washing buffer was 0.01 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.05% Tween 20. Polystyrene plates (Nunc-Immuno Plate) were coated with different concentrations of p-VIP in carbonate/bicarbonate buffer, pH 9.6, using 100 μl/well, covered with parafilm, and kept at 4 °C

TABLE I. Structure of VIP

	5		10		15		20		25	
Pig	H–His–Ser–Asp–Ala–Val–Phe–Thr–Asp–Asn–Tyr–Thr–Arg–Leu–Arg–Lys–Gln–Met–Ala–Val–Lys–Lys–Tyr–Leu–Asn–Ser–Ile–Leu–Asn–NH_2									
Cow	Val	Asn	Thr	Leu			Val	Leu	Ile	Asn
Human	Val	Asn	Thr	Leu			Val	Leu	Ile	Asn
Dog	Val	Asn	Thr	Leu			Val	Leu	Ile	Asn
Chicken	Val	Asn	Ser	Phe			Val	Leu	Val	Thr
Guinea pig	Leu	Thr	Thr	Leu			Met	Leu	Val	Asn
Dogfish	Val	Asn	Ser	Ile			Val	Ile	Leu	Ala

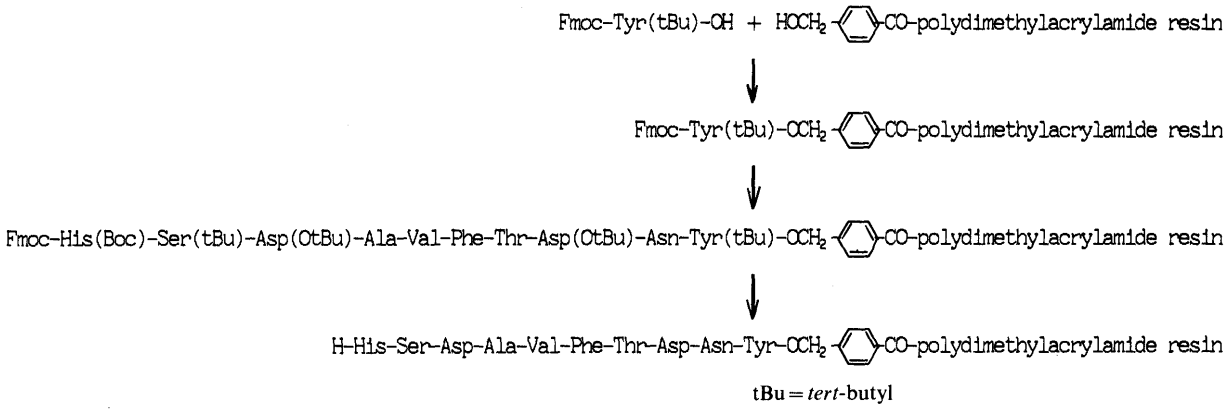

Fig. 1. Outline of the Synthesis of p-VIP(1—10)-Linked Resin

overnight. The next day, the plates were washed 4 times with the washing buffer. To block the plates, 200 μl of washing buffer containing 2% BSA was added and the plates were kept at 25 °C for 3 h. The plates were then washed, 100 μl per well of a dilution of VP-6203, was added and the plates were incubated at 25 °C for 100 min. After completion of the antigen–antibody reaction, the plates were washed 4 times. Alkaline/phosphatase-conjugated goat anti-rabbit IgG at a dilution of 1 : 1000 in assay buffer (100 μl/well) was added and the plates were incubated at 25 °C for 60 min. The plates were then washed and 100 μl per well of 0.1 M diethanolamine buffer containing 0.1% *p*-nitrophenyl phosphate was added. The optical density readings were obtained at 60 min using a Corona MTP 100 microplate reader.

Assay Procedure Solution (100 μl) of VIPs, p-VIP-fragments, h-GIP, PHI and secretin were each added to test tubes. Then 100 μl of stock antiserum at a dilution of 1 : 20000 or affinity-purified antibody at a dilution of 1 : 1600 in the assay buffer was added to all tubes and the tubes were incubated at 4 °C overnight. The plate, coated with 2 μg/ml of p-VIP mentioned above, was washed with the washing buffer 4 times and 100 μl of each antibody–antigen solution was added from the tubes and incubated for 100 min at 25 °C. The plate was then washed and 100 μl of alkaline/phosphatase-conjugated goat anti-rabbit IgG at a dilution of 1 : 1000 was added and incubated for 100 min at 25 °C. The plate was washed and 100 μl of 0.1% *p*-nitrophenyl phosphate in diethanolamine buffer, pH 9.8, was added to each well. The resulting color was determined at the wavelength of 405 nm mentioned above.

Synthesis of Porcine VIP(1—10)-Conjugated Polydimethylacrylamide Resin According to Atherton[27] *et al.*, Fmoc-Tyr(tBu)-OH was coupled to hydroxymethylbenzoylpolydimethylacrylamide resin by 1,3-diisopropylcarbodiimide in the presence of 4-dimethylaminopyridine. The N^{α}-Fmoc group was cleaved by treatment with 20% piperizine in DMF. The peptide chain was elongated using an automated peptide synthesizer (LKB Biochem. 4170) according to the principle of Dryland and Sheppard.[33] Fmoc-His(Boc)-Ser(tBu)-Asp(OtBu-Ala-Val-Phe-Thr-Asp-(OtBu)-Asn-Tyr(tBu)-OCH_2-⟨⟩-CO-polydimethylacrylamide resin, thus obtained, was treated with 20% piperizine in DMF followed by TFA to produce the desired affinity resin, H-His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-OCH_2-⟨⟩-CO-polydimethylacrylamide resin. The content of the peptide in the resin was 0.1 mmol/g at the maximum.

Affinity Separation of Specific Antibody to Porcine VIP The resin (0.5 g), synthesized as described above, was packed into a column (0.9 × 3.8 cm) and equilibrated with 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl. Next, the antiserum VP-6203 was applied to the column, and the column was washed with the above buffer. Individual fractions (1 ml each) were collected and ultraviolet(UV) absorption at 275 nm was determined. The eluted antibodies were then recycled and the column was washed with an excess of the starting buffer and 0.4 M $MgCl_2$ to remove nonspecifically bound proteins. Finally, ligand-bound antibody was eluted with 4 M $MgCl_2$ and the desired elute was dialyzed against the starting buffer (Fig. 2). The affinity purified antibody (fraction F_2 in Fig. 2) was stored in the assay buffer.

Results

Among three rabbits, two rabbits raised relatively high-titer antisera, VP-6201 and VP-6203. The titer of antiserum VP-6203 was higher than that of VP-6201, so antiserum

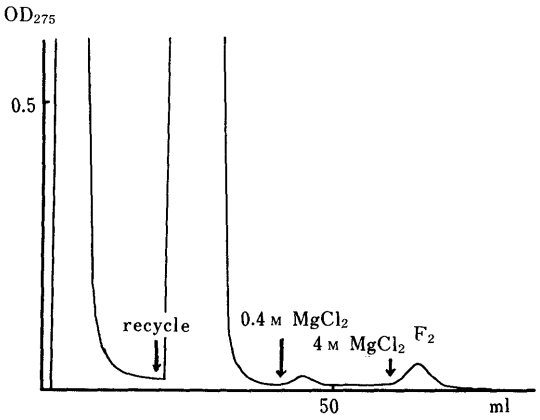

Fig. 2. Purification of Antibody VP-6203 on p-VIP(1—10)-Linked Resin

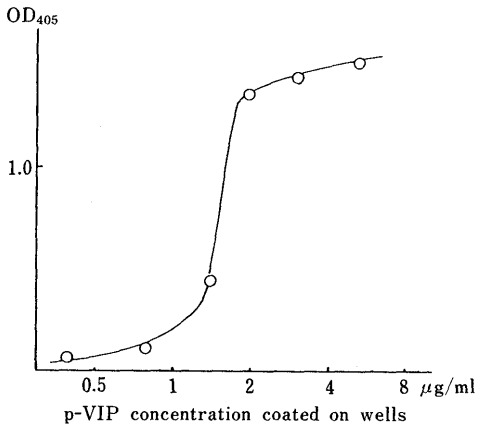

Fig. 3. Determination of Optimal Coating Concentration

VP-6203 was used for the following experiments.

Determination of Optimal ELISA Conditions Reproducibility of an ELISA is dependent on the concentration of the antigens coated on the well and of the diluted antiserum used. These parameters must be determined simultaneously to find optimal assay conditions. Figure 3 shows the absorptions obtained in the plates coated with different concentrations of p-VIP when the dilution of the antiserum VP-6203 was 1 : 40000. As the amount of p-VIP bound reached a plateau at a concentration of 2 μg/ml of p-VIP, the optimal concentration of p-VIP was determined as 2 μg/ml.

Specificity of Antiserum VP-6203 Using a microplate

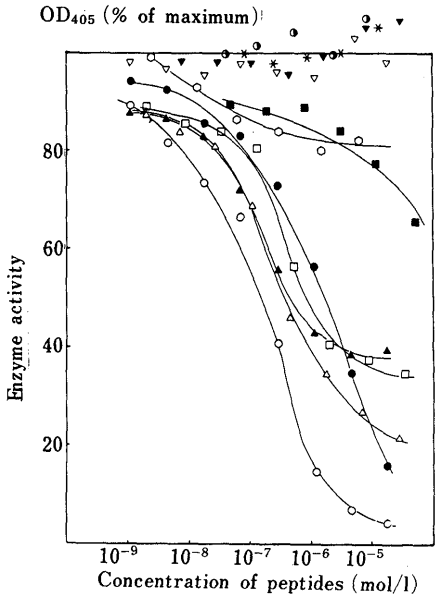

Fig. 4. Inhibition Curves of Various Peptides in ELISA by Competition between Coated p-VIP and Various Peptides for Antiserum VP-6203

p-VIP (○), c-VIP (●), GP-VIP (▽), Glu^8-p-VIP (▲), P-VIP(1—10) (○), p-VIP(11—28) (△), p-VIP(14—28) (□), p-VIP(18—28) (■), secretin (▼), PHI (✱), h-GIP (◑).

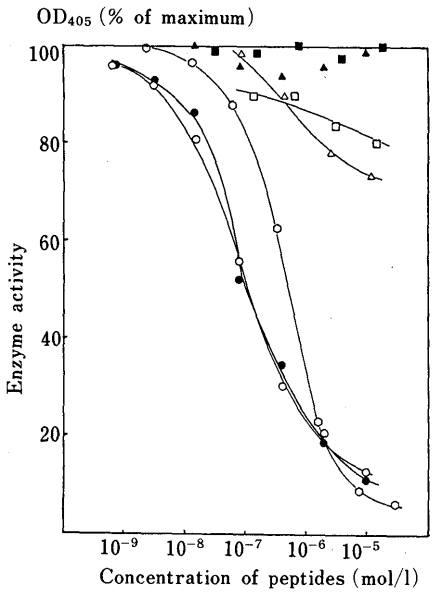

Fig. 5. Inhibition Curves of Various Peptides in ELISA by Competition between Coated p-VIP and Various Peptides for the Purified Antibody F_2

p-VIP (○), c-VIP (●), Glu^8-p-VIP (▲), P-VIP(1—10) (○), p-VIP(11—28) (△), p-VIP(14—28) (□), p-VIP(18—28) (■).

coated with 2 μg/ml of p-VIP and antiserum VP-6203 at a final dilution of 1 : 40000, sample peptides were assayed. As shown in Fig. 4, antiserum VP-6203 fully cross-reacts with p-VIP, weakly reacts with the C-terminal p-VIP fragments, c-VIP and Glu^8-p-VIP, but hardly reacts with GP-VIP, PHI, secretin, and h-GIP.

Specificity of the Affinity-Purified Antibody To obtain an N-terminal VIP specific antibody, antiserum VP-6203 was purified on polydimethylacrylamide resin conjugated with the common N-terminal decapeptide of porcine and chicken VIP as stated above, and the specificity of the purified antibody F_2 was examined. As shown in Fig. 5, the purified antibody F_2 fully reacts with p-VIP, c-VIP and the N-terminal decapeptide of p-VIP but hardly reacts with the C-terminal fragments of p-VIP and Glu^8-p-VIP.

Discussion

The antiserum VP-6203 elicited from rabbit was heterogenous and reacts not only with the N-terminal region of p-VIP but also with its C-terminal region. In order to obtain specific antibody against the N-terminal region of p-VIP, we applied an immuno-affinity purification technique, using N-terminal decapeptide (His–Ser–Asp–Ala–Val–Phe–Thr–Asp–Asn–Tyr)-linked polydimethylacrylamide resin.

As the polydimethylacrylamide resin developed for solid-phase peptide syntheses by Sheppard's group[27] is a polar resin, this gelatinous polymer is freely permeated and swollen by a wide range of solvents including water, and particularly polar aprotic media of dimethylamide type. Dimethylformamide is generally a good solvent for peptide syntheses. Thus, this resin was judged to be a suitable support for the preparation of an immuno-affinity column.

The antibody eluted from the affinity column with 4 M $MgCl_2$ (Fig. 2, F_2) was specific to p-VIP(1—10), p-VIP and c-VIP and hardly cross-reacted with Glu^8-p-VIP and C-terminal fragment of p-VIP. Thus, it was shown that affinity-purified antibody F_2 can recognize precisely the N-terminal portion of p-VIP and even the minor difference between the closely related peptides p-VIP and Glu^8-p-VIP can be distinguished by this antibody.

Thus, we were able to show that affinity chromatography on p-VIP(1—10)-linked polydimethylacrylamide resin is an effective tool to purify the specific antibody against N-terminal region of p-VIP. The method described herein will be an useful tool for isolating a specific antibody from antisera raised against structurally related peptides.

References and Notes

1) Unless otherwise stated, all amino acids with the exception of Gly are of L configuration. The following abbreviations are used: Fmoc = 9-fluorenylmethoxycarbonyl, HOBt = *N*-hydroxybenztriazole, MTPTT = 3-(3-*p*-methoxybenzylthiopropionyl)thiazolidine-2-thione, TFA = trifluoroacetic acid, GIP = glucose-dependent insulinotropic peptide, PHI = peptide histidine isoleucine, TFMSA = trifluoromethanesulfonic acid, NMM = *N*-methylmolpholine, THF = tetrahydrofuran.
2) S. I. Said and V. Mutt, *Science*, **169**, 1217 (1970).
3) S. I. Said and V. Mutt, *Eur. J. Biochem.*, **28**, 199 (1972).
4) S. I. Said and V. Mutt, *Nature* (London), **225**, 863 (1970).
5) P. J. Piper, S. I. Said and J. R. Vane, *Nature* (London), **225**, 1144 (1970).
6) G. J. Dockry, *Experientia*, **29**, 1510 (1973).
7) L. Thulin and P. Olsson, *Acta Chir. Scand., Suppl.*, **441**, 5, (1973).
8) U. T. Strunz, J. H. Walsh, S. R. Bloom, M. R. Thompson and M. I. Grossman, *Gastroenterology*, **73**, 768 (1977).
9) G. J. Krejs, R. M. Barkley, N. W. Read and J. S. Fordtran, *J. Clin. Invest.*, **61**, 1337 (1978).
10) C. Kerins and S. I. Said, *Proc. Soc. Exp. Biol. Med.*, **142**, 1014 (1973).
11) M. Schebalin, S. I. Said and G. M. Makhlouf, *Am. J. Physiol.*, **232**, E197 (1977).
12) S. I. Said and R. N. Rosenberg, *Science*, **192**, 907 (1976).
13) A. Giachetti, R. N. Rosenberg and S. I. Said, *Lancet*, ii, 741 (1976).
14) J. Fahrenkrug, *Digestion*, **19**, 149 (1979).
15) M. Bodanszky, Y. Klausner and S. I. Said, *Proc. Natl. Acad. Sci.*, **70**, 382 (1973).
16) S. I. Said and V. Mutt, *Eur. J. Biochem.*, **42**, 581 (1974).
17) A. Nilsson, *FEBS Lett.*, **60**, 322 (1975).
18) M. Carlquist, V. Mutt and H. Jornvall, *FEBS Lett.*, **108**, 457 (1979).

19) N. Itoh, K. I. Obata, N. Yanaihara and H. Okamoto, *Nature* (London), **304**, 547 (1983).
20) S. C. Wang, B. H. Du, J. Eng, M. Chang, J. D. Hulmes, Y. C. E. Pan and R. S. Yalow, *Life Sci.*, **37**, 979 (1985).
21) B. H. Du, J. Eng, J. D. Hulmes, M. Chang, Y. C. E. Pan and R. S. Yalow, *Biochem. Biophys. Res. Commun.*, **128**, 1093 (1985).
22) R. Dimaline, J. Young, D. T. Thwaites, C. M. Lee, T. J. Shuttleworth and M. C. Thorndyke, *Biochim. Biophys. Acta*, **930**, 97 (1987).
23) M. Takeyama, K. Koyama, K. Inoue, T. Kawano, H. Adachi, T. Tobe and H. Yajima, *Chem. Pharm. Bull.*, **28**, 1873 (1980).
24) H. Yajima, M. Takeyama, K. Koyama, T. Tobe, K. Inoue, T. Kawano and H, Adachi, *Int. J. Peptide Protein Res.*, **16**, 33 (1980).
25) M. Hatano, S. Funakoshi, N. Fujii, M. Takeyama, M. Yun, K. Inoue, M. Kogire, T. Tobe and H. Yajima, *Chem. Pharm. Bull.*, **36**, 3857 (1988).
26) S. R. Bloom, J. M. Polak and A. G. E. Pearse, *Lancet*, ii, 14 (1973); J. Fahrenkrug and M. O. B. Schaffalitzky, *J. Lab. Clin. Med.*, **89**, 1379 (1977); T. M. Chang, F. L. Roth, H. Tai and W. Y. Chey, *Anal. Biochem.*, **97**, 286 (1979); N. Yanaihara, M. Sakagami, H. Sato, K. Yamamoto, T. Hashimoto, C. Yanaihara, Z. Ito, K. Yamaguchi and K. Abe, *Gastroentrology*, **72**, 803 (1977).
27) E. Atherton, H. Fox, D. Harkiss, C. J. Logan, R. C. Sheppard and B. J. Williams, *J. Chem.Soc., Chem. Commun.*, **1978**, 537.
28) M. Takeyama, K. Koyama, H. Yajima, M. Moriga, M. Aono and M. Murakami, *Chem. Pharm. Bull.*, **28**, 2265 (1980).
29) S. Kiyama, K. Kitagawa, T. Akita, W. Y. Chey, A. Ayalp, A. Otsuki, S. Funakoshi, N. Fujii and H. Yajima, *Chem. Pharm. Bull.*, **33**, 3205 (1985).
30) H. Yajima, N. Fujii, K. Akaji, M. Sakurai, M. Nomizu, K. Mizuta, M. Aono, M. Moriga, K. Inoue, R. Hosotani and T. Tobe, *Chem. Pharm. Bull.*, **33**, 3578 (1985).
31) N. Fujii, W. Lee, H. Yajima, M. Moriga and K. Mizuta, *Chem. Pharm. Bull.*, **31**, 3503 (1983).
32) N. Fujii, K. Akaji, Y. Hayashi and H. Yajima, *Chem. Pharm. Bull.*, **33**, 362 (1985).
33) A. Dryland and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, **1986**, 125.