Studies on Separation of Amino Acids and Related Compounds. VIII.[1] Preparative Separation of Isomeric L-Aspartyl-L-phenylalanine Methyl Esters and Related Dipeptide Esters by Ion-Exchange Chromatography

Akira YASUTAKE, Haruhiko AOYAGI, and Nobuo IZUMIYA

Laboratory of Biochemistry, Faculty of Science, 33 Kyushu University, Higashi-ku, Fukuoka 812

(Received February 21, 1977)

Authentic peptides, α- and ω-isomers of L-aspartyl (or L-glutamyl)-L-phenylalanine (or L-tyrosine) methyl ester, were synthesized by conventional methods. Among these eight peptides, α-L-glutamyl-L-phenylalanine methyl ester showed the tendency to convert to the corresponding dipeptide anhydride even under mild conditions. The experiments on a small column of Dowex 50 cation exchange resin were carried out with a model mixture composed of the α- and ω-isomers, and the optimum conditions for the complete separation of both isomers were determined. A synthetic mixture composed of the α- and ω-isomers was prepared readily through the coupling of benzyloxycarbonyl-L-aspartic acid (or glutamic acid) anhydride with L-phenylalanine (or L-tyrosine) methyl ester and subsequent hydrogenation. The synthetic mixture was separated by the use of a large ion exchange column and each isomer was obtained in a good yield.

In 1969, Mazur *et al.* discovered that α-APM[2] was 100—200 times sweeter than sucrose.[3] Although Davey *et al.* synthesized α-APM in 1966 by the hydrogenation of Z–Asp(OBzl)–Phe–OMe,[4] the large-scale preparation of α-APM by a simpler method has been desired. As described in a previous paper,[1] we have developed a convenient procedure for the separation of the α- and β-isomers of H–Asp–His–OH by column chromatography with Dowex 50.

In the present study, we attempted to separate in quantity α- and β-APM from a synthetic mixture by similar chromatographic methods. The synthetic mixture was prepared easily as shown in Fig. 1 by coupling and subsequent hydrogenation of the resulting mixture containing Z–Asp(OH)–Phe–OMe and Z–Asp(Phe–OMe)–OH. Additionally, we intended to separate α- and β-ATM, α- and γ-GPM, and α- and γ-GTM, from corresponding synthetic mixtures

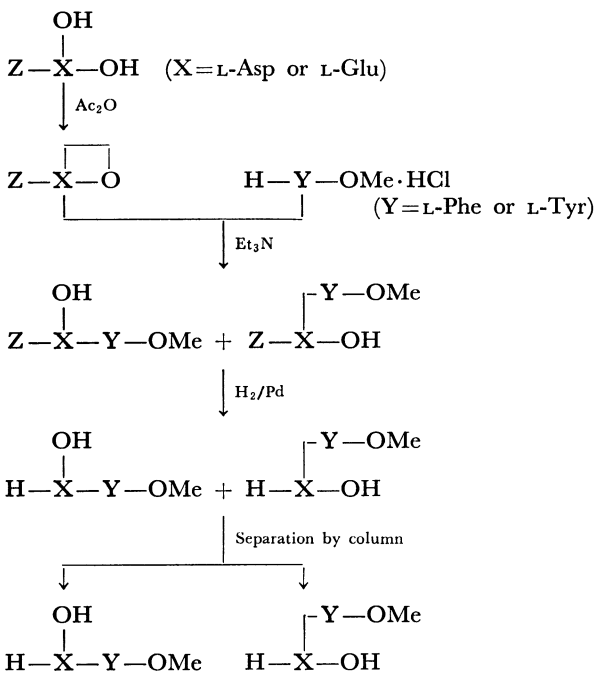

Fig. 1. Preparation of a synthetic mixture composed of α- and ω-dipeptide ester and subsequent saparation of the mixture.

which were also prepared by the coupling and hydrogenation procedure outlined in Fig. 1. These dipeptide esters are considered as interesting compounds from the standpoint of possible structure-taste relationships related to α-APM.

Experimental

TLC was carried out on Merck silica gel G, and PPC on Toyo Roshi No. 52 paper. R_f (TLC) and R_f (PPC) values are reported for the following solvent system: 1-butanol–acetic acid–pyridine–water (15 : 3 : 10 : 12, v/v). Material possessing a free terminal amino group (*e.g.*, α-APM) was detected by spraying with 0.2% ninhydrin solution in 80% ethanol, followed by heating at 100 °C. Material having blocked amino groups (*e.g.*, dipeptide anhydride) was detected by spraying with 10% H_2SO_4, followed by heating on a hot plate. $[\alpha]_D$ was measured with a Union high sensitivity polarimeter PM-71 (Kyoto).

*Authentic Peptides. Z–Asp(OBzl)–Phe–OMe (**1**), Z–Asp(OBzl)–Tyr–OMe (**3**), Z–Glu(OBzl)–Phe–OMe (**5**), Z–Glu(Phe–OMe)–OBzl (**6**), Z–Glu(OBzl)–Tyr–OMe (**7**)*: Mazur *et al.* prepared these compounds by the coupling of the Z-amino acid *p*-nitrophenyl ester, *e.g.*, Z–Asp(OBzl)–ONp, and H–Phe(or Tyr)–OMe in yields of 57—97%.[3] We prepared these compounds by the coupling of a mixed anhydride[5] of the Z-amino acid with H–Phe(or Tyr)–OMe in yields of about 70% as described for the preparation of **2**. Melting points and specific rotations of the products agreed with literature values[3] with the exceptions of mp 99—100 °C for **5** (lit, 78—80 °C), $[\alpha]_D^{20}$ −6.4° for **7** (*c* 2, MeOH) (lit, −44°). We observed that the synthesis of Z–Asp(OH)–OBzlNO_2[6] was easier than that of Z–Asp(OH)–OBzl;[7] therefore, the intermediate compounds (**2** and **4**) were prepared as follows.

*Z–Asp(Phe–OMe)–OBzlNO_2 (**2**)*: To a chilled solution of Z–Asp(OH)–OBzlNO_2 (4.02 g, 10 mmol) and triethylamine (1.4 ml, 10 mmol) in tetrahydrofuran (25 ml) was added isobutyl chloroformate (1.31 ml, 10 mmol) at −10 °C. After 15 min, a chilled solution of H–Phe–OMe·HCl (2.16 g, 10 mmol) and triethylamine (1.4 ml, 10 mmol) in chloroform (20 ml) was added. The reaction mixture was left to stand at room temperature overnight, evaporated *in vacuo*, and the oily residue was dissolved in ethyl acetate (300 ml). The solution was washed successively with 4% $NaHCO_3$, 2% HCl and water, dried (Na_2SO_4), and evaporated. The crystals were collected with the aid of ether; yield, 3.84 g (68%); mp 162—164 °C; $[\alpha]_D^{20}$ −40.9° (*c* 1, MeOH).

Found: C, 61.73; H, 5.16; N, 7.28%. Calcd for $C_{29}H_{29}O_9N_3$: C, 61.80; H, 5.19; N, 7.46%.

*Z-Asp(Tyr-OMe)-OBzlNO$_2$ (**4**)*: This compound was prepared from Z-Asp(OH)-OBzlNO$_2$ (10 mmol) and H-Tyr-OMe·HCl (10 mmol), as described for the preparation of **2**; yield, 4.18 g (72%); mp 163—164 °C; $[\alpha]_D^{20}$ −40.4° (*c* 1, MeOH).

Found: C, 60.04; H, 5.06; N, 7.19%. Calcd for $C_{29}H_{29}O_{10}N_3$: C, 60.10; H, 5.04; N, 7.25%.

*Z-Glu(Tyr-OMe)-OBzl (**8**)*: This compound was prepared from Z-Glu(OH)-OBzl[8] (10 mmol) and H-Tyr-OMe·HCl (10 mmol), as described above; yield, 3.50 g (64%); mp 149 °C; $[\alpha]_D^{20}$ −9.2° (*c* 1, MeOH).

Found: C, 65.54; H, 6.05; N, 5.07%. Calcd for $C_{30}H_{32}O_8N_2$: C, 65.68; H, 5.88; N, 5.11%.

α-APM, β-APM, α-ATM, β-ATM, α-GPM, γ-GPM, α-GTM: These compounds were prepared by the hydrogenation of **1**—**7**, as described for the preparation of γ-GTM. Their specific rotations agreed with literature values[3] with the exceptions of $[\alpha]_D^{20}$ +6.1° (*c* 2, H_2O) for β-APM (lit, +4°)[3] and $[\alpha]_D^{20}$ +15.8° for γ-GPM (*c* 2, H_2O) (lit, +21°).[3]

γ-GTM: A solution of **8** (0.55 g, 1 mmol) in a mixture of methanol (12 ml) and acetic acid (3 ml) was treated with hydrogen in the presence of palladium black, and the filtrate was evaporated *in vacuo*. The resulting solid was recrystallized from hot water; yield, 0.30 g (93%); mp 197—199 °C; $[\alpha]_D^{20}$ +6.9° (*c* 2, H_2O), $[\alpha]_D^{20}$ +39.2° (*c* 2, AcOH); R_f (TLC) 0.68, R_f (PPC) 0.54.

Found: C, 55.40; H, 6.23; N, 8.51%. Calcd for $C_{15}H_{20}O_6N_2$: C, 55.55; H, 6.22; N, 8.64%.

*H-α-Glu-Phe-OH Anhydride (**9**)*: α-GPM (62 mg, 0.2 mmol) was dissolved in methanol (10 ml) previously saturated with ammonia, and the solution was allowed to stand at room temperature for 2 days. The solvent was removed by evaporation, the residue was dissolved in water (10 ml), and the solution was acidified with 1 M HCl (0.2 ml). After the solution was evaporated to dryness, the resulting crystals were collected by the filtration with the aid of cold water; yield, 42 mg (71%); mp 235—236 °C (dec); $[\alpha]_D^{20}$ +28.8° (*c* 1, AcOH); R_f (TLC) 0.75.

Found: C, 60.54; H, 5.93; N, 10.18%. Calcd for $C_{14}H_{16}O_4N_2$: C, 60.56; H, 5.84; N, 10.14%.

Column Chromatography. A column was packed with Dowex 50X8 (200—400 mesh) equilibrated with a specified eluting solvent. The eluting solvents used were aqueous pyridinium acetate at different concentrations and pHs as follows; S_1=0.1 M at pH 4, S_2=0.2 M at pH 4, S_3=0.2 M at pH 5, S_4=1 M at pH 5. A mixture of dipeptide esters was applied to a column and eluted with one of these eluting solvents at room temperature. An aliquot (0.5 ml) of each fraction was subjected to a test to determine the amount of ninhydrin-positive material present using the Yemm and Cocking method.[9]

Results and Discussion

Column Chromatography of a Model Mixture Containing Isomeric Dipeptide Esters. Preliminary experiments on a model mixture were performed as follows with a small column in order to find the optimum conditions for the separation. A model mixture was prepared by dissolving 0.01 mmol each of α- and β-APM in water (0.5 ml). Other model mixtures (α- and β-ATM, α- and γ-GPM, and α- and γ-GTM) were prepared similarly. The model mixture was applied to a column (0.9×20 cm) and eluted with a specified eluting solvent,

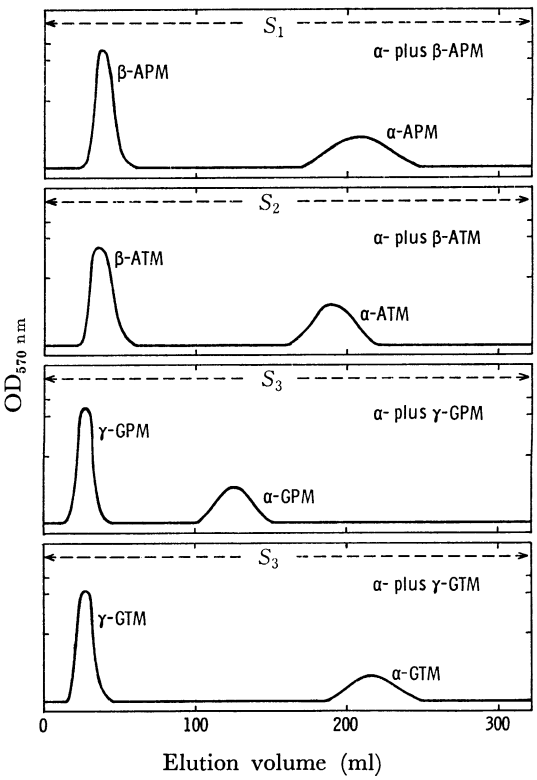

Fig. 2. Chromatography on a 0.9×20 cm column of Dowex 50 of model mixtures. For example, α- plus β-APM represents a model mixture composed of α-APM and β-APM (total, 0.02 mmol).

2-ml fractions being collected at a flow rate of 20 ml/h. The peptide in each fraction was identified by means of TLC or PPC after concentration of the corresponding fraction by evaporation.

We applied pyridinium acetate in various concentrations and pHs to determine necessary separation conditions of the α- and ω-isomers in a model mixture. An example with satisfactory results is shown in Fig. 2. When higher concentrations of pyridinium acetate and more alkaline pHs were used, the distance of separation between the α- and ω-isomers was diminished.

As seen in Fig. 2, the α-isomer was eluted more slowly than the corresponding ω-isomer in every case. We can give no definite explanation for this phenomenon, but we can point out that the same phenomenon was observed in the previous study in which H-β-Asp-His-OH or H-γ-Glu-His-OH was eluted faster than the corresponding α-dipeptide.[1]

Preparative Separation of α-APM and β-APM from a Synthetic Mixture. A synthetic mixture was prepared according to Fig. 1 and each isomer was separated with a large column. To a solution of Z-Asp-OH anhydride[10] (2.49 g, 10 mmol) in tetrahydrofuran (10 ml) was added a solution of H-Phe-OMe·HCl (2.38 g, 11 mmol) and triethylamine (1.54 ml, 11 mmol) in chloroform (20 ml) while stirring at room temperature. After stirring overnight, the reaction mixture was evaporated to dryness and the residue was dissolved in a mixture of methanol (40 ml) and acetic acid (10 ml). The solution was treated with hydrogen in the presence of palladium black. The filtrate was evaporated to dry-

Table 1. Elution data for preparative separation of synthetic mixture

Material	Elution solvent	Eluate volume (ml)	Portion for evaporation (ml)	Peptide present
Mixture of				
α- and β-APM	S_1	0—1000	580—980	β-APM
	S_3	1000—2000	1340—1760	α-APM
α- and β-ATM	S_2	0—1000	660—900	β-ATM
	S_3	1000—2000	1620—1900	α-ATM
α- and γ-GTM	S_3	0—1000	300—520	γ-GTM
	S_4	1000—1600	1400—1700	α-GTM
α- and γ-GPM	S_3	0—500	300—420	γ-GPM
	S_4	500—1200	900—1100	α-GPM[a]

a) α-GPM was converted into H-Glu-Phe-OH anhydride mostly after evaporation of the portion (900—1100 ml).

Table 2. Yields and physical constants of peptides

Peptide	Yield (%)	Melting point (°C dec)	$[\alpha]_D^{20}$ (c 1)		R_f	
			H_2O	AcOH	TLC	PPC
β-APM	32	181—182	+6.2°	+40.4°	0.69	0.67
α-APM	37	239—242	+0.2°	+33.0°	0.74	0.72
β-ATM	31	200—202	+14.8°	+38.4°	0.65	0.55
α-ATM	34	266—268	+5.2°	+36.2°	0.69	0.61
γ-GTM	27	201—202	+7.0°	+39.0°	0.69	0.55
α-GTM	34	215—217	+15.6°	+46.0°	0.72	0.76
γ-GPM	27	160—162	+1.2°	+38.4°	0.69	0.71
H-α-Glu-Phe-OH anhydride	31	234—236	—[a]	+28.6°	0.76[b]	
Authentic α-GPM[c]		105—109	+1.2°	+45.2°	0.74[b]	0.80

a) H-α-Glu-Phe-OH anhydride was slightly soluble in water, hence an accurate determination of $[\alpha]_D$ in water was difficult. b) When chloroform-methanol-acetic acid (25 : 5 : 1, v/v) was used as a solvent on TLC, R_f values were 0.54 for H-α-Glu-Phe-OH anhydride and 0.14 for authentic α-GPM. c) Data of this peptide synthesized by the conventional method are given as reference values.

ness, and the resulting powder (a synthetic mixture) was used in the next step.

The powder was dissolved in S_1 (20 ml) and put on a column (2.7×50 cm) of Dowex 50. The sample was eluted with 1000 ml of eluant S_1 at a flow rate of 40 ml/h, and 20-ml fractions were collected. Then, the eluant was changed to S_3 in order to shorten elution time, and 1000 ml of S_3 was eluted. The eluting solvents used in this study are summarized in Table 1. The elution pattern is shown in Fig. 3 as a representative example among similar experiments. A portion of 580—980 ml was evaporated in a bath at about 50 °C *in vacuo*, the evaporations being repeated several times by the additions of water.

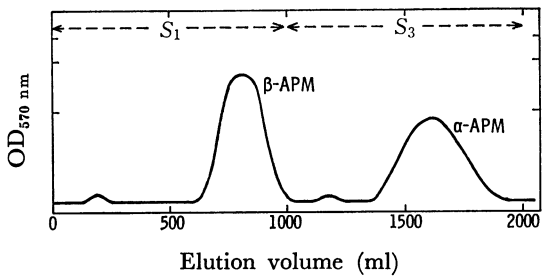

Fig. 3. Chromatography on a 2.7×50 cm column of Dowex 50 of a synthetic mixture (10 mmol) composed of α-and β-APM.

The residual product was recrystallized from hot water; yield of β-APM, 0.95 g (32% from Z-Asp-OH anhydride), the data being shown in Table 2. A portion of 1340—1760 ml was treated similarly; yield of α-APM, 1.09 g (37% from Z-Asp-OH anhydride).

As mentioned above, we could isolate pure α- and β-APM in good yields from a synthetic mixture by the use of a column with ion-exchange resin. Ariyoshi *et al.* prepared a similar synthetic mixture by the coupling of L-aspartic acid anhydride (1 equivalent) and H-Phe-OMe (4 equivalents); this procedure is advantageous because it avoids the hydrogenation step as seen in Fig. 1, but suffers from the contamination of the product with excess H-Phe-OMe and polymers that are produced by over reaction of L-aspartic acid anhydride.[11] The same investigators reported the isolation of α-APM hydrochloride, which was less soluble than β-APM hydrochloride, by means of fractional crystallization from their synthetic mixture.[11]

Preparative Separations of α-ATM and β-ATM, and α-GTM and γ-GTM, from Each Synthetic Mixture. Each synthetic mixture was prepared from Z-Asp-OH anhydride (10 mmol) and H-Tyr-OMe·HCl (11 mmol), and from Z-Glu-OH anhydride[12] (10 mmol) and H-Tyr-OMe·HCl (11 mmol), respectively, as described for the preparation of the synthetic mixture containing α- and β-APM. Subsequently, each mixture was subjected to column chromatography as described for the

isolation of α- and β-APM, the data of eluting solvents being summarized in Table 1. Each isomer was obtained in yields of 0.97 g (31%) for β-ATM, 1.06 g (34%) for α-ATM, 0.87 g (27%) for γ-GTM and 1.09 g (34%) for α-GTM. The physical constants are summarized in Table 2.

Preparative Separation of H–α-Glu–Phe–OH Anhydride and γ-GPM from a Synthetic Mixture. The synthetic mixture prepared from Z–Glu–OH anhydride (10 mmol) and H–Phe–OMe·HCl (11 mmol) was treated on a column as described above. A portion of the eluate (300—420 ml) yielded 0.84 g (27%) of pure γ-GPM (see Tables 1 and 2). It was observed that a portion of the 900—1100 ml fraction contained α-GPM; however, the residual powder obtained after evaporation of this fraction was a mixture of H–α-Glu–Phe–OH anhydride as a major product and α-GPM as a minor product. The residual powder was collected by filtration with the aid of cold 0.1 M HCl to remove α-GPM, and subsequently washed with cold water; yield of H–α-Glu–Phe–OH anhydride, 0.91 g (31%); mp 234—236 °C (dec) (see Table 2).

At the beginning stages of this experiment, we had assumed that the product with mp 234—236 °C was either Pyroglu–Phe–OMe or H–α-Glu–Phe–OH anhydride. Since the results of IR and NMR of the product indicated no presence of methyl ester group, we compared the product with authentic H–α-Glu–Phe–OH anhydride(**9**), and were able to identify the product as the anhydride. We discovered that the H–α-Glu–Phe–OH anhydride was produced during evaporation of the eluate and the amount produced was dependent upon the temperature of the bath and the extent of reduced pressure during the evaporation. For example, the residual powder after evaporation at 30 °C with a good aspirator at 15—20 mmHg was a 3 : 7 mixture of the anhydride to α-GPM. Since these results suggested the lability of α-GPM compared with other dipeptide esters, we carried out the following experiments.

Stability of Dipeptide Esters. Authentic dipeptide methyl esters (eight kinds) were stored in bottles at room temperature for about 4 months, and each was examined by TLC and PPC. The results indicated that α-GPM was converted mostly into H–α-Glu–Phe–OH anhydride and only a small amount of the original peptide ester remained. On the contrary, the seven other peptide esters remained unchanged after 4 months.

The experiment was also carried out in solution. 25 μmol of each of the pure authentic dipeptide esters (eight kinds) was dissolved in S_4 (1 ml), and the solution was allowed to stand at 25 °C. Aliquots withdrawn at selected intervals up to 6 days were subjected to TLC, and examined for the appearance of new peptides. After 6 days, an incubation solution of α-GPM contained an appreciable amount (about 20%) of H–α-Glu–Phe–OH anhydride, and each solution of α-APM, α-ATM, and α-GTM also contained some amounts (5—10%) of new products which are presumably the corresponding dipeptide anhydrides. On the contrary, each solution of ω-dipeptide esters was stable producing no new product.

No definite explanation for the marked tendency of α-GPM to convert to the corresponding anhydride can be given at present. Recently, Furda *et al.* examined the relative stabilities of α-APM and its hydrochloride in aqueous solution, and detected the presence of H–α-Asp–Phe–OH anhydride as a major product.[13]

Sweetness Evaluation. The sweetness of these compounds was organoleptically determined by panel evaluation in our laboratory according to the literature.[14] α-APM and α-ATM, isolated from the synthetic mixture, had intense sweet tastes similar to the corresponding authentic samples. Other β-aspartylpeptide esters and all glutamylpeptide esters, including γ-GTM which was prepared newly in this study, showed weak bitter tastes. Mazur *et al.* reported the intense sweet tastes of α-APM and α-ATM, and the bitter tastes of many β-aspartyl and glutamylpeptide esters.[3]

References

1) M. Hirata, K. Noda, and N. Izumiya, *Bull. Chem. Soc. Jpn.*, **45**, 1290 (1972).
2) Standard amino acid symbols denote the L-configuration. One letter abbreviations used are: A, Asp; G, Glu; P, Phe; T, Tyr; M, methyl ester. Thus, *e.g.* α-APM represents α-L-aspartyl-L-phenylalanine methyl ester, γ-GTM γ-L-glutamyl-L-tyrosine methyl ester. Other abbreviations used are: Z, benzyloxycarbonyl; OBzl, benzyl ester; OBzl-NO_2, *p*-nitrobenzyl ester; TLC, thin-layer chromatography; PPC, paper chromatography.
3) R. H. Mazur, J. M. Schlatter, and A. H. Goldkamp, *J. Am. Chem. Soc.*, **91**, 2684 (1969).
4) J. M. Davey, A. H. Laird, and J. S. Morley, *J. Chem. Soc., C*, **1966**, 555.
5) J. R. Vaughan, Jr. and R. L. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1952).
6) E. Schröder and E. Klieger, *Justus Liebigs Ann. Chem.*, **673**, 208 (1964).
7) T. Hayakawa, Y. Fujiwara, and J. Noguchi, *Bull. Chem. Soc. Jpn.*, **40**, 1205 (1967).
8) E. Klieger and H. Gibian, *Justus Liebigs Ann. Chem.*, **655**, 195 (1962).
9) E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955).
10) W. D. John and G. T. Young, *J. Chem. Soc.*, **1954**, 2870.
11) Y. Ariyoshi, T. Yamatani, N, Uchiyama, Y. Aoki, and N. Sato, *Bull. Chem. Soc. Jpn.*, **46**, 1893 (1973).
12) W. J. LeQuesne and G. T. Young, *J. Chem. Soc.*, **1950**, 1954.
13) I. Furda, P. D. Malizia, M. G. Kolor, and P. J. Vernieri *J. Agric. Food Chem.*, **23**, 340 (1975).
14) Y. Ariyoshi, N. Yasuda, and T. Yamatani, *Bull. Chem. Soc. Jpn.*, **47**, 326 (1974).