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A Solid Support for Affinity Chromatography that Covalently Binds Thiol Groups via a Cleavable Connector Arm[1]

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Preparation of an agarose derivative (MPE-agarose) containing a maleimido group which is attached to agarose via a cleavable phenyl ester linkage is described. MPE-agarose was shown to react with the thiol groups in glutathione, bovine serum albumin, bovine hemoglobin, and yeast and rabbit muscle glyceraldehyde 3-phosphate dehydrogenase. Treatment of the resulting agarose-linked compounds for 10 min with 1 M hydroxylamine (pH 7) resulted in the cleavage of the phenyl ester linkage, and release of the maleimido derivative of the compound from the gel. In the case of hemoglobin and glyceraldehyde 3-phosphate dehydrogenase noncovalent interactions between the gel and the released protein lowered the amount of protein which dissolved in the hydroxylamine solution upon cleavage of the phenyl ester linkages. Noncovalently absorbed protein could be removed from the gel, however, by washing the gel with 2 M guanidine hydrochloride after treatment with hydroxylamine. Derivatives of MPE-agarose should prove useful in affinity chromatography and immunoabsorption where it is difficult to elute material bound to conventional affinity supports.

Solid supports which covalently bind specific functional groups in proteins and small organic molecules via cleavable linkages should prove useful in affinity chromatography and immunoabsorption when elution of the absorbed material is difficult. Material strongly absorbed to an immobilized ligand could be removed from such a support by breaking a cleavable linkage between the support and the immobilized ligand. This procedure would of course result in release of the entire complex from the support. Well-established techniques such as dialysis, ion exchange chromatography, and electrophoresis could be used, however, to separate and further purify the components of the released complex. Such macromolecular reagents containing cleavable linkages and reactive groups directed toward specific functional groups in proteins might also prove useful in the separation of proteins with different functional groups as well as in the isolation of active site peptides, after partial digestion of the reagent-linked protein. Recently, we have described the preparation and properties of a macromolecular reagent (SEPE-agarose)[4] for covalently linking ligands containing amino groups to agarose via phenyl ester linkages which can be broken without destroying disulfide and carbohydrate groups that are present in many proteins (1).[5] We now report the prep-

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[4] Abbreviations used: BSA, bovine serum albumin; GAPDH, glyceraldehyde 3-phosphate-dehydrogenase; MPE-agarose, the maleimide attached to agarose via phenyl ester linkages that forms upon reaction of SEPE-agarose with *N*-(6-aminohexyl)maleimide; NEM, *N*-ethylmaleimide; protein-linked MPE-agarose, the agarose derivative that forms upon reaction of MPE-agarose with protein; SEPE-agarose, the *N*-succinimidyl ester attached to agarose via phenyl ester linkages which is depicted in Eq. [1]; tlc, thin-layer chromatography.

[5] The phenyl ester linkage and the recently reported thiol ester linkage (see ref. 14) have the advantage over the previously reported cleavable disulfide, diazo, and vicinal hydroxyl linkages, in that these ester linkages can be cleaved under mild conditions that don't result

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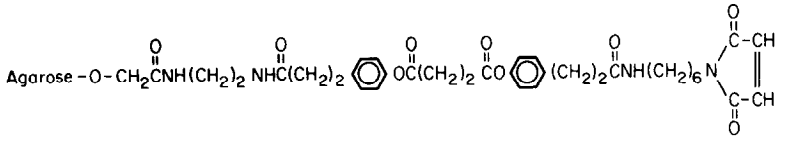
aration and properties of a derivative of SEPE-agarose, MPE-agarose, which can be used to link thiol-containing compounds to agarose via cleavable phenyl ester linkages.[6]



MPE-Agarose

MATERIALS AND METHODS

BSA, GSH, and hemoglobin (bovine) were obtained from Calbiochem. Myoglobin (horse heart) was obtained from Sigma Chemical Company. Yeast and rabbit muscle GAPDH were obtained from Boehringer–Mannheim and Sigma, respectively. Silica gel-coated plastic sheets (Polygram SILG-UV_{254}) for tlc were from Brinkman Instruments. *S*-Succinylcysteine was prepared according to the method of Calam and Waley (2). Methyl methanethiolsulfonate was prepared according to the method of Smith *et al.* (3). 1,6-Diaminohexane monohydrochloride was prepared by neutralizing 1,6-diaminohexane with 1 eq. of 1 M HCl and removing the water under reduced pressure.

N-(6-Aminohexyl)maleimide was prepared by refluxing a solution of maleic anhydride (9.8 g, 0.1 mol) and 1,6-diaminohexane monohydrochloride (15.2 g, 0.1 mol) in acetic acid (200 ml) for 7 h, and removing the solvent under reduced pressure. The resulting residue was taken up in 100 ml of 0.5 M HCl, evaporated to dryness, and extracted six times with 50 ml ether. The ether-insoluble material was dissolved and precipitated repeatedly (three to six times) with methanol–ether to yield a white crystalline solid. This material was chromatographed on a silica gel column (4 × 40 cm) which was eluted with chloroform–ethanol–1 M HCl (15:5:0.4). The fractions which showed a single spot (visualized by spraying with 1% $KMnO_4$ in 10% Na_2CO_3) on tlc (chloroform–ethanol–1 M HCl, 15:5:0.4) were combined and recrystallized from chloroform–methanol to give 2.4 g (10% yield) of crystalline material, mp 160–62°C (corr). An analytical sample (mp 166–68°C, corr) was prepared by recrystallizing the chromatographed material twice from methanol–ether.

Infrared spectrum (KBr, 3450, 2938, 1700, 1600, 1418, 1122 cm^{-1}). H NMR spectrum (60 MHz, CD_3OD) δ6.82 (2H, s, olefinic), 3.28 (2H, t, J = 3 Hz, $-CH_2$), 2.99 (2H, t, J = 3 Hz, $-CH_2$), 1.46 (8H, m, $-CH_2$).

Anal. Calcd for $C_{10}H_{17}N_2O_2Cl$ (232.71): C, 51.61; H, 7.36; N, 12.04; Cl, 15.23. Found: C, 51.60; H, 7.30; N, 11.87; Cl, 15.08 (Galbraith Laboratories, Knoxville, Tenn.).

MPE-agarose was prepared by stirring a solution of *N*-(6-aminohexyl)maleimide hydrochloride (232 mg, 1 mmol) in 10 ml 0.1 M phosphate buffer, pH 7.4, for 1 h with 3 ml of SEPE-agarose. The supernatant solution was removed by centrifugation and the gel washed three times with 10 ml of 0.1 M phosphate buffer, pH 7.4. Unreacted succinimidyl ester groups present in the gel were destroyed by reacting the gel for 30 min with 10 ml of 1 M ethanolamine in 0.1 M phosphate buffer, pH 7.4. The gel was then washed three times with 6 ml of each of the following solutions: 0.1 M phosphate buffer, pH 7.4; 1 M phosphate buffer, pH 7.4; 0.01 M HCl; and dioxane. The resulting material, MPE-agarose, was stored under dioxane.

Reaction of MPE-agarose with GSH was affected by washing 0.3 ml MPE-agarose three times with 3 ml of 0.1 M phosphate buffer, pH 7.4, and then stirring the washed gel for 30 min with 3 ml of a solution of GSH (1 mM) in 0.1 M phosphate buffer, pH 7.4, containing 5 mM EDTA. The amount of GSH which had reacted with the gel was estimated by two methods. In one method the amount of the reacted GSH was determined from the difference in the concentration of dissolved GSH before and after reaction with the gel. In this method, the concentration of GSH was estimated spectrally from the color reaction of GSH with 5,5′-dithiobis(2-nitrobenzoic acid) in 0.15 M phosphate buffer, pH 7.2 (4). In the other method of analysis, the gel was separated from the supernatant solution and washed three times with 3 ml of 0.1 M phosphate buffer, pH 7.4, and then the amount of GSH covalently bound to the gel was determined from the amount of glutamic acid, *S*-succinylcysteine, and glycine obtained upon acid hydrolysis of the gel at 100°C for 72 h in the presence of 2 ml of 6 M HCl containing 5 μl mercaptoethanol (5, 6).

Reaction of proteins with MPE-agarose was effected by stirring 3 ml of a (2 mg/ml) protein solution with 0.1

in reduction of disulfide bonds or oxidation of glycosyl residues which are present in many proteins. A cleavable crosslinking agent containing alkyl ester linkages has also been described (see Ref. (15)). Cleavage of the alkyl ester linkages with hydroxylamine is less facile, however.

[6] MPE-agarose binds thiols covalently through its maleimido group. Maleimido-containing solid supports without cleavable linkages have been previously described (see Ref. (16)).

to 0.3 ml MPE-agarose for 15 min to 4 h as described for the reaction of GSH with MPE-agarose. The gel was then washed three times with 4 ml of each of the following solutions: 0.1 M phosphate buffer (pH 7.4), 2 M guanidine hydrochloride, water, 1 M phosphate buffer (pH 7.4) and water to give protein-linked MPE-agarose. In the case of hemoglobin, the washes contained 1 mM methyl methanethiolsulfonate. The protein content of protein-linked MPE-agarose was estimated from amino acid analysis (5) after acid hydrolysis of the gel.

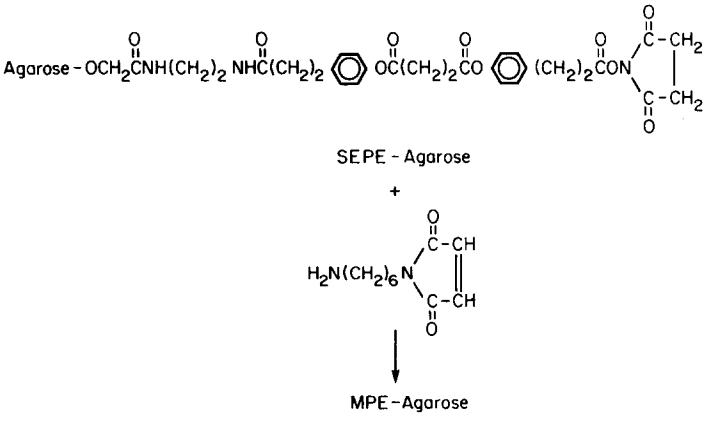
Control experiments were carried out in which the reactive sulfhydryl groups in proteins were blocked prior to reaction of the protein solution with the gel. The sulfhydryl groups in BSA and hemoglobin were blocked by a 1-h treatment at pH 7.4 of the protein (in 0.1 M phosphate buffer, pH 7.4, containing 5 mM EDTA) with a 10-fold excess of an aqueous solution of methyl methanethiolsulfonate. The reactive sulfhydryl groups in GAPDH were blocked by a 1-h treatment of GAPDH (in 0.1 M phosphate buffer, pH 7.4, containing 5 mM EDTA) with 2 mM NEM in the presence of 5% ethanol, followed by another 1-h treatment with 0.3 mM *N*-(6-aminohexyl)maleimide hydrochloride. After appropriate dilution with 0.1 M phosphate buffer (pH 7.4) containing 5 mM EDTA, the protein solution (2 mg/ml) was reacted with MPE-agarose and the gel was washed as described for proteins that had not been treated with the thiol reagents. The amount of the protein remaining on the washed gel was determined from the amino acid content of the gel after acid hydrolysis.

Release of protein from protein-linked MPE-agarose was effected by stirring for 10 min with 3 ml of a freshly prepared solution of 1 M hydroxylamine, pH 7.0. The amount of the protein present in the hydroxylamine supernatant solution was estimated from the amino acid content after dialysis and acid hydrolysis of the solution. In order to estimate the amount of the protein remaining on the gel after treatment with hydroxylamine, the gel was washed three times with 3 ml each of the following solutions: water, 0.1 M phosphate buffer (pH 7.4), 2 M guanidine hydrochloride, water, 1 M phosphate buffer, (pH 7.4), and water; and the amount of the protein remaining on the gel was estimated from the yield of amino acids after acid hydrolysis of the gel.

The S-succinylcysteine content of BSA released from BSA-linked MPE-agarose was measured in order to determine the extent of reaction between the thiol group of BSA and the maleimido group of MPE-agarose. BSA-linked MPE-agarose was prepared by reacting 10 ml of BSA (2 mg/ml) in 0.1 M phosphate buffer (pH 7.4) containing 5 mM EDTA for 4 h with 1 ml of MPE-agarose as described above for the reaction of proteins with the gel. BSA was released from the BSA-linked MPE-agarose by treatment for 6 h with 8 ml of a buffer consisting of imidazole and glycine at pH 7.4. The supernatant solution was concentrated to 2 ml by ultrafiltration and the concentrated solution was subjected to gel filtration on a column (2 × 40 cm) of Bio-Gel P-2 (100–200 mesh) which was eluted with 0.01 M phosphate buffer, pH 7.0, containing 1 mM mercaptoethanol. The fractions in the void volume were hydrolyzed in 6 M HCl at 110°C for 72 h and subjected to amino acid analysis (5, 6).

RESULTS AND DISCUSSION

The solid-phase maleimide (MPE-agarose) was prepared by reacting SEPE-agarose with *N*,6-aminohexylmaleimide according to Eq. [1]:



[1]

MPE-Agarose was observed to react rapidly with the thiol group of GSH. In the presence of 1 mM GSH at pH 7.4, reaction with MPE-agarose was complete within the 5–10 min necessary to obtain complete diffusion of GSH into the MPE-agarose.

This result is consistent with previous studies which have demonstrated that maleimides react rapidly with thiol groups (7–9) and are well suited for crosslinking thiol-containing compounds (10, 11). A rough estimate of the expected reactivity of a maleimido group under the conditions used for the reaction of GSH with MPE-agarose can be obtained from kinetic data reported by Gorin *et al.* (9). Their study of the pH dependence of the rate of reaction of cysteine with NEM indicates that in the presence of 1 mM cysteine, the half-life of NEM should be about 0.2 s.

The MPE-agarose preparations used in this work had a capacity to bind 2.3 ± 0.5 μmol GSH/ml gel. The amount of GSH which bound to the gel as estimated from the depletion of the thiol content of a GSH solution upon its reaction with MPE-agarose was within 10% of the GSH that became covalently linked to MPE-agarose as estimated from the acid hydrolysate of reacted gel that had been extensively washed in order to remove any noncovalently bound GSH. This result suggests that all of the GSH that was lost from the reaction mixture formed a stable covalent linkage with the gel. Had any GSH become bound to the gel through noncovalent interactions or through unstable covalent bonds, GSH would be expected to be lost from the gel during the washing procedure. In such a case, the amount of GSH lost from the reaction mixture would be greater than the GSH content of the washed gel. Acid hydrolysis of the reacted gel liberated equivalent amounts of glutamic acid, *S*-succinylcysteine, and glycine. This result indicates that all of the GSH in the washed gel resulted from the addition of the thiol group of GSH to the olefinic bond of the maleimido group of MPE-agarose. Had any GSH become linked to the gel as a result of other reactions, the amount of *S*-succinylcysteine obtained from the acid-hydrolyzed gel would have been less than that of glutamic acid and glycine.

It should be borne in mind, however, that the maleimido group of NEM is capable of reacting with imidazolyl and amino groups. Since these reactions are much slower than the reaction of the maleimide with thiol groups (12, 6, 14), it is not surprising that little or no GSH became linked to the MPE-agarose via a reaction of its amino group. A protein, on the other hand, could conceivably contain one or more highly reactive amino groups which become covalently linked to MPE-agarose via Michael addition to the olefinic bond of the maleimido group or via nucleophilic displacement on one of the carbonyl carbon atoms of the maleimido group. These possibilities were investigated in our studies of the reaction of MPE-agarose with thiol-containing proteins, wherein the effect of blocking the thiol group on the amount of protein incorporated in the gel was determined. As shown in Table I, blocking the thiol groups in a protein prior to its reaction with MPE-agarose (controls) decreased the binding of the protein to MPE-agarose by 5- to 20-fold. The binding of protein to the gel in the control experiments probably resulted from reaction of amino groups of the protein with the maleimido group of MPE-agarose or from noncovalent binding of the protein to the gel or both. An attempt was made to minimize the effect of noncovalent binding in this work by washing reacted gels (both control and experimental) with 2 M guanidine hydrochloride in order to facilitate removal of noncovalently bound material from the gel.

The interaction of MPE-agarose with horse heart myoglobin, a protein devoid of thiol groups, also was studied in an effort to assess further the specificity of MPE-agarose as a thiol reagent. Surprisingly, acid hydrolysis and amino acid analysis of MPE-agarose that had been reacted with a commercial sample of horse heart myoglobin indicated substantial incorporation of protein in the gel. The relative amounts of the amino acids found in the acid hydrolysate of the gel were markedly different from that of myoglobin, however. For example, the amount of aspartic acid in the hydrolysate of the gel corresponded to 57 nmol myoglobin/ml of gel, whereas the amount of isoleucine corresponded to 13 nmol myoglobin/ml of gel. Interestingly, the amount of amino acids found in the acid hydrolysate of the gel was reduced by 10- to 20-fold when the myoglobin was incubated with the thiol blocking reagent methyl methanethiolsulfonate prior to its reaction with the gel. These

TABLE I

BINDING AND RELEASE REACTIONS OF PROTEINS WITH HMPE-AGAROSE[a]

Protein	Time of reaction	Amount incorporated[b] (nmol/ml gel)		Amount Released[c] (nmol/ml gel)		Amount remaining[d] (nmol/ml gel)	
		Experimental	Control	Experimental	Control	Experimental	Control
BSA	15 min	35	3.5	34		1.6	
	2 h	88	4.6				
Hemoglobin (bovine)	4 h	79	11	25	3.7	20	2.2
	4 h	63		27[e]		15 (34[f])	
GAPDH (yeast)	4 h	34	1.0	1.3		4.0	1.0
GAPDH (rabbit muscle)	4 h	46	11	<1.0 (29[g])		11	2.0

[a] Amounts of hemoglobin and GAPDH listed in this table are molar amounts of tetrameric protein.

[b] Determined from the amino acid content after acid hydrolysis of the gel. The control experiment was carried out using protein whose thiol groups were blocked by reaction with methyl methanethiosulfonate or by reaction with NEM and *N*-(6-aminohexyl)maleimide (in the case of GAPDH) prior to reaction with the gel.

[c] After reaction with 1 M hydroxylamine, pH 7.0, for 10 min, the amount of released protein was determined from the amino acid content after acid hydrolysis of the supernatant solution.

[d] Subsequent to treatment with hydroxylamine, the gels were washed three times with 3 ml each of the following solutions: water; 0.1 M phosphate buffer, pH 7.4; 2 M guanidine hydrochloride; water; 1 M phosphate buffer and water. In order to determine the amount of the gel-bound protein, the gel was hydrolyzed with 6 M HCl at 100°C for 72 h and the amino acid content of the hydrolysate determined.

[e] After treatment with hydroxylamine solution the gel was stirred for 15 min with 2 ml of water and the aqueous solution was combined with the hydroxylamine supernatant solution. The combined solution was used for acid hydrolysis.

[f] The gel remaining after the hydroxylamine and water treatment was acid hydrolyzed without any more washings. The acid hydrolysate was used to determine the amino acid content.

[g] In the first wash with guanidine hydrochloride as determined spectrophotometrically.

results indicate that the myoglobin was impure and contained a thiol protein which reacted with MPE-agarose. Solid supports such as MPE-agarose should prove useful in the detection, removal, and characterization of minor constituents of proteins such as the thiol-containing protein in the myoglobin preparation.

Previous studies of SEPE-agarose (1) have shown that the phenyl ester linkages of the gel are stable in neutral solutions in the absence of nucleophiles, but are easily cleaved by nucleophiles such as hydroxylamine and imidazole at neutral pH values. Table I illustrates the near quantitative release of BSA from MPE-agarose upon treatment with hydroxylamine. In other experiments treatment with imidazole also was found to release BSA from the gel. Upon acid hydrolysis, the released BSA yielded 1.0 mol of *S*-succinylcysteine/mol of BSA. The stoichiometric yield of *S*-succinylcysteine and the low amount of BSA incorporated in the control experiments support the view that the BSA was linked to the gel primarily via its single thiol group.

The difference between the amount of protein incorporated in the gel and that remaining on the gel after hydroxylamine treatment indicates that the hydroxylamine treatment resulted in the release of over 75% of the gel-bound hemoglobin and GAPDH. It should be noted, however, that the amounts of hemoglobin and GAPDH that were observed in the hydroxylamine solution were less than one would expect from the difference in the protein contents of the gel before and after treatment with

hydroxylamine (Table I). This discrepancy was found to be due to the removal of protein from the gels when the gels were washed with 2 M guanidine hydrochloride subsequent to the hydroxylamine treatment. Thus, the first guanidine hydrochloride wash after hydroxylamine treatment of a GAPDH-linked gel contained more than 60% (29 nmol/ml of gel) of the GAPDH that had been originally linked to the gel (Table I). Removal of protein from hydroxylamine-treated gels during the procedure used to wash the gels was also indicated by an increase from 15 to 34 nmol/ml in the hemoglobin content of a gel when the washing procedure was omitted (Table I). Only protein which had been previously covalently linked to the gel was removed by the guanidine hydrochloride washes which followed hydroxylamine treatment. All gels were washed with 2 M guanidine hydrochloride prior to treatment with hydroxylamine in order to remove protein which became noncovalently bound to the gel during the initial reaction between protein and the maleimido group. The procedure, which was used to wash gels prior to determinations of their initial protein contents, might have caused some dissociation of the tetrameric proteins and loss of subunits which were not linked covalently to the gel. Such a process cannot account for the loss of protein from the gel after hydroxylamine treatment, however, since repetition of the procedure initially used to wash the gels did not result in the release of protein unless it was preceded by treatment of the gel with hydroxylamine.

Thus, the low extent of release of GAPDH from MPE-agarose in the presence of hydroxylamine was probably due to noncovalent interactions between the protein and the MPE-agarose. It should be realized that similar difficulties may be encountered in the dissolution of substances which are either insoluble in the medium used to cleave the phenyl ester linkages or substances which interact noncovalently with the gel. Dissolution of such substances for further purification may also be facilitated by guanidine hydrochloride, or other agents such as detergents or simply buffered solutions of low ionic strength.

The approach described here to prepare the maleimido derivative of SEPE-agarose should be applicable to the preparation of similar derivatives of SEPE-agarose which react with other functional groups in proteins.

REFERENCES

1. SINGH, P., LEWIS, S. D., AND SHAFER, J. A. (1979) *Arch. Biochem. Biophys.* **193**, 284–293.
2. CALAM, D. H., AND WALEY, S. G. (1963) *Biochem. J.* **86**, 226–231.
3. SMITH, D. J., MAGGIO, E. T., AND KENYON, G. J. (1975) *Biochemistry* **14**, 766–771.
4. ELLMAN, G. (1959) *Arch. Biochem. Biophys.* **82**, 70–77.
5. SPACKMAN, D. H., STEIN, W. H., AND MOORE, S. (1958) *Anal. Chem.* **30**, 1190–1206.
6. SMYTH, D. G., BLUMENFELD, O. O., AND KONIGSBERG, W. (1964) *Biochem. J.* **91**, 589–595.
7. GREGORY, J. D. (1955) *J. Amer. Chem. Soc.* **77**, 3922–3923.
8. LEE, C. C., AND SAMUELS, E. R. (1961) *Canad. J. Chem.* **39**, 1152–1154.
9. GORIN, G., MARTIC, P. A., AND DOUGHTY, G. (1966) *Arch. Biochem. Biophys.* **115**, 593–597.
10. TROMMER, W. E., FRIEBEL, K., KILTZ, H. H., AND KOLKENBROCK, H. J. (1977) *in* Advances in Experimental Medicine and Biology (Friedman, M., ed.), Vol. 86, Pt A, pp. 185–187, Plenum, New York.
11. KITAGAWA, T., AND AIKAWA, T. (1976) *J. Biochem.* **79**, 233–236.
12. SMYTH, D. G., NAGAMATSU, A., AND FRUTON, J. S. (1960) *J. Amer. Chem. Soc.* **82**, 4600–4604.
13. BREWER, C. F., AND RIEHM, J. P. (1967) *Anal. Biochem.* **18**, 248–255.
14. BROWN, R. J., SWAISGOOD, H. E., AND HORTON, H. R. (1979) *Biochemistry* **18**, 4901–4906.
15. ABDELLA, P. M., SMITH, P. K., AND ROYER, G. P. (1979) *Biochem. Biophys. Res. Commun.* **87**, 734–742.
16. MANECKE, G., AND VOGT, H. G. (1978) *Pure Appl. Chem.* **50**, 655–666.