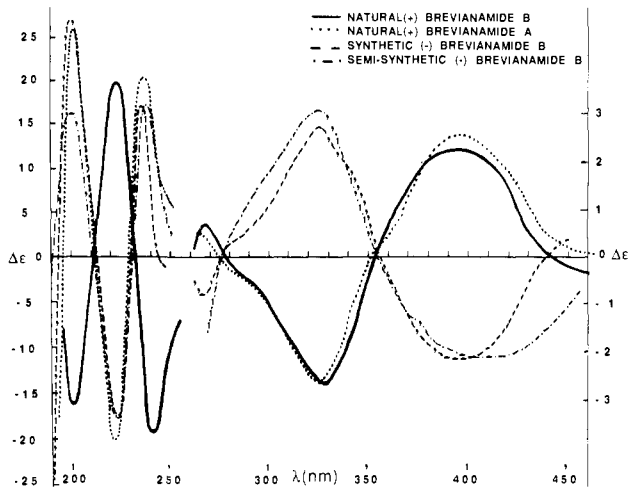

Figure 1. CD spectra of **1** and **2** recorded in trifluoroethanol between 200 and 250 nm and in 2.5% formic acid/dichloromethane between 250 and 450 nm.

stereochemistry at the spiroindoxyl stereogenic center which is (*R*)- for brevianamide A *and* (*R*)- for *natural* brevianamide B. The CD spectra between 250 and 450 nm for these two substances are virtually identical even though they are diastereomers belonging to unique enantiomorphic groups. The UV spectra of these substances show an absorbtion at ~400 nm which is attributable to the indoxyl chromophore.[1]

If the Birch biosynthetic pathway[1] is correct with respect to the structures of intermediates **5**–**7**, our results require that (1) both enantiomorphs of **7** are synthesized by *Penicillium brevicompactum* in *unequal* amounts and either (2) two distinct oxidases having unique *enantioselectivity* for (+)-**7** and (−)-**7** must also have *opposite diastereoselectivity* delivering oxygen from the more and less hindered faces of **7** (furnishing **8** and **9**, respectively) resulting in optically pure **1** and **2** or (2) that a single oxidase recognizes only the binding orientation of the indole moiety, delivering oxygen from the (*R*)-face of each enantiomer of **7**. The contention that the oxidation of **7** to **1** or **2** is enzyme-mediated is supported by the following observation. An authentic, synthetic sample of the proposed shunt metabolite (−)-**7** was prepared by removal[6] of the *p*-methoxybenzyl group from N-9 of the corresponding synthetic[3,7] derivative. Allowing this compound to stand in ethyl acetate solution exposed to air for several days resulted in a myriad of decomposition products of which no identifiable trace of either **1** or **2** could be detected. However, m-CPBA oxidation of (−)-**7** followed by exposure of the incipient hydroxyindolenine to NaOMe in methanol gave in high yield (exclusively) (−)-brevianamide B. Thus, unlike deoxybrevianamide A (**3**) which autoxidizes[1] to (−)-**2**, compound **7** does *not* autoxidize to either **1** or **2** implicating a specific, enzyme-mediated process. Attempts to identify **7** in culture extracts of *Penicillium brevicompactum* were completely unsuccessful. While this does not rule out the possibility that **7** is a short-lived, tightly enzyme-bound intermediate that is not excreted into the culture medium, further experiments are required to prove the validity of this reasonable biosynthetic scheme. The relative proportions of brevianamide A and B produced suggests that **7** is produced in partially racemic form. The failure to detect **7**, particularly (+)-**7** which precedes **2**, supports the notion that both enantiomorphic precursors to **1** and **2** are produced in unequal amounts and are completely consumed by the oxidase(s). An interesting mystery that remains is to elucidate the mechanism for the formation of the two enantiomorphic series, regardless of the validity of structure **7**.

The above facts lead to the conclusion that *Penicillium brevicompactum* has evolved genes encoding for enantio- and diastereodivergent pathways specifically for the biosynthetic production of **1** and **2** regardless of the structural uncertainties of the intermediates following **4**. Planar, achiral intermediate **6** would nicely accommodate the occurrence of the two enantiomeric series; a single oxidase displaying complete (*R*)-facial selectivity toward the indole, or two distinct enantio- and diastereoselective oxidases, would then effect a resolution producing the two optically pure diasteromers **1** and **2**. Experiments aimed at validating the intermediacy of **5**, **6**, and **7** as shunt metabolites and elucidating the nature of the oxidase(s) are in progress in these laboratories.

Acknowledgment. We thank the National Institutes of Health (CA 43969) and the Colorado State University Agricultural Experiment Station (part of USDA SAES Western Project W-122) for financial support of this work. Prof. Robert W. Woody (CSU) is acknowledged for assistance in obtaining the CD spectra. R.M.W. acknowledges The Alfred P. Sloan Foundation, Eli Lilly Co., and the National Institutes of Health (Research Career Deveopment Award) for additional Fellowship support.

(5) Specific optical rotations for these substances further support the CD data: synthetic **2** $[\alpha]_D^{25} = -124°$ (*c* 0.77, CH_2Cl_2/2.5% HCO_2H); natural **2** (from *Penicillium brevicompactum* directly) $[\alpha]_D^{25} = +124°$ (*C* 0.77, CH_2Cl_2/2.5% HCO_2H); semisynthetic **2** (derived from **1** via oxidation of **3**) $[\alpha]_D^{25} = -124°$ (*c* 0.77, CH_2Cl_2/2.5% HCO_2H). The synthetic material (**2**) was shown to be >99% ee.

(6) Williams, R. M.; Kwast, E. *Tetrahedron Lett.* **1989**, *30*, 451.

(7) Reference 3, compound no. 16.

Studies of the Inactivation of General Acyl-CoA Dehydrogenase by Racemic (Methylenecyclopropyl)acetyl-CoA: New Evidence Suggesting a Radical Mechanism of This Enzyme-Catalyzed Reaction

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Received November 25, 1988

General acyl-CoA dehydrogenase (GACD) is a flavin (FAD) dependent enzyme which catalyzes the first step of β-oxidation converting a straight chain fatty acyl thioester substrate **1** to the corresponding α,β-enolyl-CoA product **2**.[1] Studies of this desaturation step are of particular mechanistic interest, since it involves the rupture of two kinetically stable C–H bonds. Evidence has accumulated supporting a C_α deprotonation as the initial step of this dehydrogenation.[1] However, the mechanism of the subsequent transfer of reducing equivalents from the carbanion **3** to the oxidized flavin is still disputable.[1,2] The commonly accepted route consists of C_β–H expulsion from **3** and then hydride addition to FAD yielding, in a net trans elimination, the α,β-enolyl-CoA **2** and the fully reduced flavin (eq 1).[1] While this mechanism appears to be quite feasible, it should be kept in mind that oxidized flavin is a poor hydride acceptor.[3] Oxidation of the carbanion **3** via a one-electron route forming a transient radical species **4** and a semiquinone flavin is a compelling alternative (eq 2).[1d-3] In fact, formation of flavin radical upon addition of substrate to acyl-CoA dehydrogenase has indeed been noted.[4,5] Several recent

(1) (a) Thorpe, C.; Matthews, R. G.; Williams, C. H. *Biochemistry* **1979**, *18*, 331. (b) Ghisla, S.; Thorpe, C.; Massey, V. *Biochemistry* **1984**, *23*, 3154. (c) Pohl, B.; Raichle, T.; Ghisla, S. *Eur. J. Biochem.* **1986**, *160*, 109. (d) Ghisla, S. In *Flavins and Flavoproteins*; Bray, R. C., Engel, P. C., Mayhen, S. G., Eds.; Walter de Gruyter & Co.: Berlin, 1984; p 385 and references cited therein.

(2) (a) Massey, V.; Ghisla, S. In *Biological Oxidations, 34, Mosbach Colloquim*; Sund, H., Ullrich, V., Eds.; Springer: Berlin, 1983; p 114. (b) Bruice, T. C. In *Advances in Chemistry Series 191: Biomimetic Chemistry*; American Chemical Society: Washington, DC, 1980; p 89.

(3) (a) Farng, O. L.; Bruice, T. C. *J. Chem. Soc., Chem. Commun.* **1984**, 185. (b) Hemmerich, P.; Massey, V.; Ferner, H. *FEBS Lett.* **1977**, *84*, 5.

(4) McKean, M. C.; Sealy, R. C.; Frerman, F. E. In *Flavins and Flavoproteins*; Massey, V., Williams, C. H., Eds.; Elsevier: Amsterdam, 1982; p 614. When the natural acceptor, electron-transfer flavoprotein (ETF), is present with excess substrate, the yield of this radical is increased by 2-fold.

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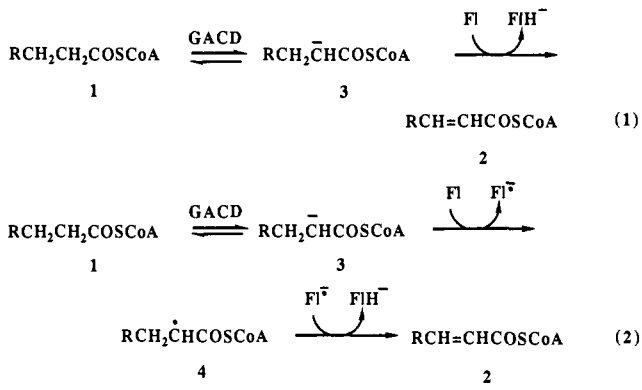
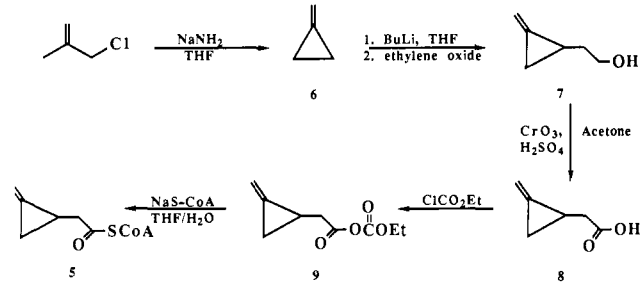


attempts to solve this long disputed problem have involved the use of spectroscopic reaction kinetic techniques coupled with the use of deuterated substrates or deazaflavin analogues.[1b-d] Although the results of these experiments have been interpreted to support the hydride-transfer mechanism (eq 1), it is not yet certain whether substitution of deazaflavin for flavin induces a change in the enzyme mechanism.

In an effort to gain new insights into the GACD-catalyzed reaction, we decided to re-examine the well-known inhibition of this enzyme by (methylenecyclopropyl)acetyl-CoA (MCPA-CoA) (**5**).[6] This methylenecyclopropane derivative is a metabolite of hypoglycine A[7] which is the causative agent of the Jamaican vomiting sickness.[8] The molecular basis of this inhibition is believed to proceed with an initial C_α anion formation, followed by ring fragmentation, and then covalent modification of the flavin coenzyme.[6] Although the crucial ring cleavage leading to inactivation has been proposed to be a direct anion-induced process, it may also be envisaged as occurring via a transient α-cyclopropyl radical intermediate. Since the rearrangement of α-cyclopropyl radicals to the ring-opened alkyl radicals is extremely rapid with equilibrium greatly favoring the unconstrained acyclic partner,[9] the consequent ring-opening step is expected to be nonstereospecific if, indeed, an α-cyclopropyl radical forms along the reaction coordinate.

In order to test this contention and to probe the electron-transfer process (1e⁻ vs 2e⁻ route) mediated by general acyl-CoA dehydrogenase, we have chemically synthesized MCPA-CoA in racemic form. As depicted in Scheme I, the key intermediate, methylenecyclopropylethanol **7**, was prepared by the incubation of ethylene oxide with a methylene cyclopropane anion which was derived from methylenecyclopropane **6**[11] and *n*-butyllithium in THF at 0 °C (65% yield). Oxidation of this alcohol with Jones reagent in acetone gave carboxylic acid **8** (90%). Condensation of **8** with ethyl chloroformate to form a mixed anhydride **9**[10] followed by the coupling of coenzyme A in aqueous THF solution (pH 8–8.5) afforded the desired MCPA-CoA (**5**). The crude

Scheme I



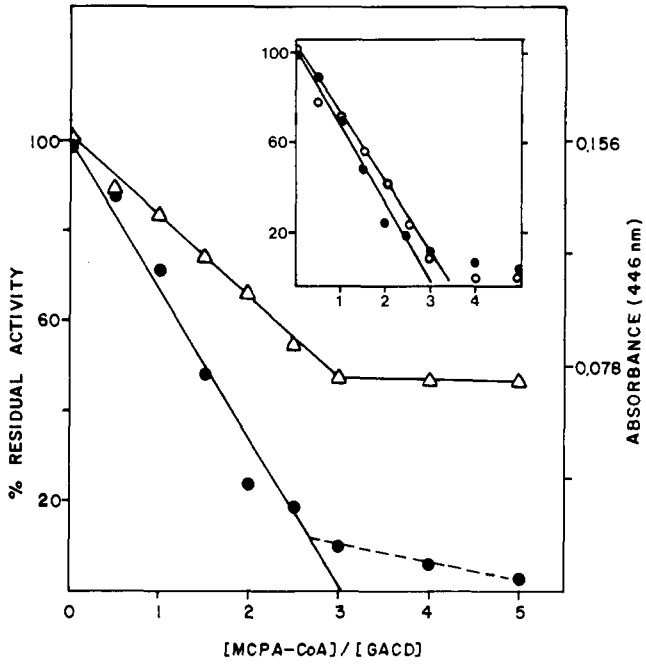


Figure 1. Effect of MCPA-CoA on the flavin absorbance (446 nm) and the catalytic activity of GACD. The purified enzyme (14 nmol) in 60 mM potassium phosphate buffer (pH 7.5) was titrated aerobically with successive addition of aliquots of MCPA-CoA (14 nmol each time). Intermediate spectra were recorded after absorbance changes were completed (about 15 min), and the residual activity was assayed concurrently according to a procedure of Thorpe.[13] The inset shows the percentage of residual oxidized flavin absorbance and the percentage of residual activity vs the ratio of MCPA-CoA to enzyme: ((△), absorbance at 446 nm; (●), % residual activity; (○), % residual absorbance at 446 nm = $[A_t - A_f]/[A_i - A_f]$; A_t, absorbance at time t; A_f, final absorbance; A_i, initial absorbance).

MCPA-CoA was chromatographed on a DEAE-Sephadex A-25 column and eluted with a 0.2–0.6 M NaCl (in 0.1 mM HCl) linear gradient.[12] Fractions containing MCPA-CoA gave a negative result to the nitroprusside test[13] and had an A_{232}/A_{260} ratio of 0.5–0.54. The pooled fractions were then desalted by Bio-Gel P-2 chromatography (0.1 mM HCl) and lyophilized. The overall yield from **6** was 45%.

Following the method developed by Wenz et al.,[6] the inactivation of GACD[14] was analyzed by successive titration with aliquots of the racemic MCPA-CoA (**5**). The inactivation was monitored by both the bleaching of the flavin chromophore (λ_{max} 446 nm) and the loss of enzyme activity. As shown in Figure 1, a plot of the residual activity observed under aerobic conditions vs total equivalents of racemic MCPA-CoA added gave a partition ratio of 2 which is identical with the literature value[6a] obtained

(5) Forming a covalent intermediate via the addition of the carbanion **3** to the flavin or the collapse of the radical pair (**4** and flavin semiquinone) prior to transferring the reducing equivalent has also been postulated,[1d] but the low acidity of the β-H in the adduct and the observation of internal transfer of β-H to the flavin[1b,d] have rendered these routes unlikely.

(6) (a) Wenz, A.; Thorpe, C.; Ghisla, S. *J. Biol. Chem.* **1981**, *256*, 9809. (b) Ghisla, S.; Wenz, A.; Thorpe, C. In *Enzyme Inhibitors*; Brodbeck, U., Ed.; Verlag Chim: Weinheim, 1980; p 43. (c) Abeles, R. H. In *Enzyme Activated Irreversible Inhibitors*; Seiler, N., Jung, M. J., Koch-Weser, J., Eds.; Elsevier-North Holland: Amsterdam, 1978; p 1.

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(8) (a) Tanaka, K. In *Handbook of Clinical Neurology*; Vinken, P. J., Bruyn, G. W., Eds.; Elsevier-North Holland: Amsterdam, 1979; Vol. 37, Chapter 17, p 511. (b) Stuart, K. L. In *Hypoglycin*; Kean, E. A., Ed.; Academic: New York, 1975; p 39.

(9) (a) Stubbe, J. *Biochemistry* **1988**, *27*, 3893, and references cited therein. (b) Griller, D.; Ingold, K. U. *Acc. Chem. Res.* **1980**, *13*, 317.

(10) Belcher, M. *Methods Enzymol.* **1981**, *72*, 404.

(11) (a) Köster, R.; Arora, S.; Binger, P. *Liebigs Ann. Chem.* **1973**, 1219. (b) Köster, R.; Arora, S.; Binger, P. *Angew. Chem., Int. Ed. Engl.* **1969**, *8*, 205. Compound **6** is commercially available from Fluka Chemical Corporation.

(12) Williamson, G. Ph.D. Thesis, 1985, University of Sheffield.

(13) Stadtman, E. R. *Methods Enzymol.* **1957**, *3*, 931.

(14) The GACD used in this study was purified from pig kidney to homogeneity based on a procedure described by Thorpe (Thorpe, C. *Methods Enzymol.* **1981**, *71*, 366).

from incubation with enzymatically derived optically active MCPA-CoA.[15] This finding clearly indicates that both of the stereoisomers of MCPA-CoA are competent inhibitors for GACD, and therefore the inactivation is nonstereospecific.[16] Since the C_β–H cleavage of the trans dehydrogenation step is well established to be pro-*R* specific,[17] the lack of stereospecificity of bond rupture at C_β of MCPA-CoA found in our inactivation study strongly suggests that this ring-opening step leading to inactivation is not enzyme-controlled. Hence, such ring fragmentation is likely a spontaneous event, induced by an α-cyclopropyl radical.[18] Since the rearrangement of α-cyclopropyl radicals to the ring-opened alkyl radicals are extremely rapid,[9,19] the ring cleavage may bypass the chiral discrimination imposed by the enzyme.

Figure 1 also revealed that the extent of flavin modification parallels the loss of enzyme activity, although the rate of inactivation is slightly faster than that of bleaching. Furthermore, the bleaching of the flavin chromophore levels off first while small loss of enzyme activity continues. These phenomena may be ascribed to the existence of a minor inactivation pathway involving alkylation of the apoprotein as previously surmised.[1a,6b,20] Since none of the common amino acids possess an electrophilic center, such alkylation, if it indeed occurs concurrently with flavin modification, stands against the nucleophilic ring-opening mechanism.[21]

Thus, study of the MCPA-CoA-mediated mechanism-based inhibition of general acyl-CoA dehydrogenase seems to favor a radical-initiated process. Since this enzyme is expected to operate via a single mechanism, the mechanistic insights derived from the inhibition study provide compelling evidence arguing for a radical mechanism of general acyl-CoA dehydrogenase-catalyzed reaction. If the unusual structure of the inhibitor has led the enzyme to proceed through a different mechanism than it would normally follow, the aforementioned findings connote, at the very least, that general acyl-CoA dehydrogenase is capable of mediating one-electron oxidation–reduction.[22]

(15) MCPA-CoA was generally prepared from hypoglycin via L-amino acid oxidase mediated deamination and H_2O_2 induced decarboxylation to yield methylenecyclopropane acetic acid followed by thioester formation catalyzed by acyl-CoA synthetase.[6]

(16) However, this preliminary observation contradicts Baldwin's recent report (Baldwin, J. E.; Parker, D. W. *J. Org. Chem.* **1987**, *52*, 1475) in which they concluded that the C_1 epimer of MCPA-CoA shows no significant influence on the inactivation of enzyme by MCPA-CoA itself, and, thus, the inactivation is stereospecific. Since the MCPA-CoA used in our study was highly purified, quantitation of the inhibitor concentration was more accurate.

(17) (a) Biellmann, J. F.; Hirth, C. G. *FEBS Lett.* **1970**, *9*, 55. (b) Biellmann, J. F.; Hirth, C. G. *FEBS Lett.* **1970**, *9*, 335. (c) Bucklers, L.; Umani-Ronchi, A.; Retey, J.; Arigoni, D. *Experientia* **1970**, *26*, 931.

(18) The X-ray structure of this enzyme (Kim, J. P.; Wu, J. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 6677) has revealed that there is enough room for the acyl-CoA substrate at either side of the flavin ring. However, a recent stereochemical study showed that H transfer in this enzyme is via the *re* face of the flavin (Manstein, D. J.; Pai, E. F.; Schopfer, L. M.; Massey, V. *Biochemistry* **1986**, *25*, 6807) indicating that only *re* side binding is catalytically productive. This also excludes the possibility that enantiomers of MCPA-CoA could bind to opposite sides of the flavin to trigger the observed flavin modification since the initial binding and the subsequent α-proton abstraction shared by normal catalysis and MCPA-CoA-mediated inactivation should follow the same course.

(19) The additional ring strain imposed by the attached exocyclic double bond in MCPA-CoA and the capability of the product to stabilize the transient radical post ring cleavage may render the ring-opening step more rapid and apparently enzyme independent.

(20) Crane, F. L.; Mii, S.; Hauge, J. G.; Green, D. E.; Beinert, H. *J. Biol. Chem.* **1956**, *218*, 701.

(21) However, a report exists claiming no apoprotein modification during this inactivation (Zeller, H. D.; Ghisla, S. In *Flavins and Flavoproteins*; Edmondson, D. E., McCormick, D. B., Eds.; Walter de Gruyter: Berlin, 1987; p 161). Confirmation of our preliminary results would require the incubation of GACD with properly labeled [^{14}C]MCPA-CoA of high purity.

(22) The flavin-MCPA-CoA adduct, upon the treatment of excess $Fe^{III}CN$ under anaerobic conditions, gave an absorbance maximum around 650 nm which is quite different from the original flavin semiquinone absorption at 560 nm of this protein.[1a] The fact that the inhibitor–flavin adduct can be reoxidized by $Fe^{III}CN$ strongly suggests that this adduct is a reduced flavin species and the observed absorption maximum should be informative in comparison with appropriate model systems. Since the modified flavin is known to be very unstable when isolated,[6,21] study of its spectroelectrochemical properties holds promise for directly deducing the general structural features of the modified cofactor in its intact form at the active site of the inactivated enzyme.

Acknowledgment. This work is supported by National Institute Health Grants GM 25344 (M.T.S.) and GM 35906/GM 40541 (H.W.L.). H.W.L. also thanks the Camille & Henry Dreyfus Foundation for a grant awarded to Distinguished New Faculty in Chemistry and the American Cancer Society for a Junior Faculty Research Award.

Role of Ring Strain and Steric Hindrance in a New Method for the Synthesis of Macrocyclic and High Polymeric Phosphazenes

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Received November 4, 1988

At present, the most general synthetic route to poly(organophosphazenes) **1** from small molecule cyclophosphazenes involves the ring-opening polymerization of the cyclic trimer $[NPCl_2]_3$ (**2**) to give the soluble high polymeric reactive intermediate $[NPCl_2]_n$ (**3**), which then functions as a substrate for chlorine atom replacement by a wide variety of organic nucleophiles (route A).[1-6] A second route to phosphazene polymers involves the condensation polymerization of *N*-silylphosphoranimines, a method that provides direct access to a range of alkyl- or arylpolyphosphazenes.[7] However, in principle, an alternative method of synthesis can be visualized that involves the introduction of organic and organometallic side groups at the cyclic trimer level (to give **4**)[8] followed by the ring-opening polymerization of these species (route B). The advantage of this route is that the substitution chemistry would be carried out on small molecule cyclic species rather than on the more sensitive macromolecular intermediates.[9]

Although many halogeno cyclic phosphazenes have been polymerized,[8,10] until now all attempts to polymerize fully substituted cyclic trimers of type **4** to high molecular weight materials have been unsuccessful.[11-13]

Recent synthetic advances have provided access to species in which strain is imparted to the phosphazene ring by means of transannular metallocenyl units.[14,15] Such ring strain is known to enhance the ease of polymerization of cyclophosphazenes that also bear halogen substituents.[16,17] We have now found that ring

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(3) Allcock, H. R.; Kugel, R. L. *Inorg. Chem.* **1966**, *5*, 1716.
(4) Allcock, H. R. *Angew. Chem., Int. Ed. Engl.* **1977**, *16*, 147.
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(6) *Inorganic and Organometallic Polymers*; Zeldin, M., Wynne, K. J., Allcock, H. R., Eds.; ACS Symposium Series; American Chemical Society: Washington, DC, 1988; Vol. 360, pp 250–282.
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(16) Allcock, H. R.; Lavin, K. D.; Riding, G. H. *Macromolecules* **1985**, *18*, 1340.
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