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Decarboxylation of Cinnamic Acids by *Saccharomyces cerevisiae*

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The stereochemistry of the decarboxylation of 3,4-dimethoxycinnamic acids by *Saccharomyces cerevisiae* and the enzymatic specificity with respect to the substrate structure were studied. This reaction proceeds with retention of configuration at the side-chain double bond as well as enzymatic specificity for the (*E*) configuration. The influence of substituents in the α and β positions was also studied. Hypotheses on the reaction mechanism were proposed.

INTRODUCTION

Oxygenated cinnamic acids are widely distributed in higher plants, where they act as precursors of phenylpropanoid compounds (lignins, coumarins, flavonoids, etc.) (*1*). Some of them, those bearing an hydroxyl group in *p*-position, are converted by many microorganisms into the corresponding styrenes through a nonoxidative decarboxylation (*2–6*). Thus styrenes and their hydrogenated products (*p*-ethylphenols) occur in a wide variety of beverages including beer, wine, and whiskey (*7–9*), being produced by yeast fermentation of plant material (*10, 11*). It is well recognized that even in trace amounts, ring-oxygenated styrenes markedly contribute to the organoleptic properties (flavor) of beverages (*9*).

Our attention has been focused on a selected strain of *Saccharomyces cerevisiae*, which was found to be able to decarboxylate 3,4-dimethoxycinnamic acid, as well as ferulic acid. In this paper, we report the chemical investigation of the decarboxylation process in order to clarify the reaction stereochemistry (*12*) and the enzymatic specificity with respect to the substrate structure.

RESULTS AND DISCUSSION

The overall stereochemistry of cinnamic acids decarboxylation was shown to be with retention of configuration at side-chain double bond, as indicated in Scheme 1 (*3, 12*).

This rested on the following spectroscopic evidence. The nmr spectrum of (*Z*)-3,4-dimethoxy-[β-^{2}H]styrene, obtained from decarboxylation of (*E*)-3,4-dimethoxy-[α-^{2}H]cinnamic acid, exhibited a pattern of signals in agreement with

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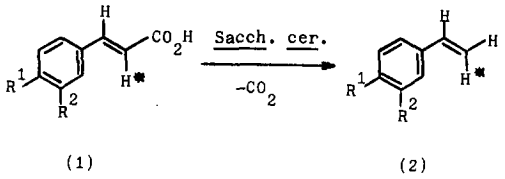


a) R^1=OH R^2=H
b) R^1=OCH_3 R^2=H
c) R^1=OH R^2=OCH_3
d) R^1=R^2=OCH_3

SCHEME 1

the theoretical one, calculated assuming that (a) $J_{HD} = (\gamma_D/\gamma_H) J_{HH}$ and (b) the chemical shifts are not affected by deuterium substitution (*13*). In addition, such a pattern appeared clearly distinguishable from that of synthetic (*E*)-3,4-dimethoxy-[β-^{2}H]styrene.

Enzyme specificity of the decarboxylation reaction toward substrate was investigated by administering a number of cinnamic acids to resting cells of *S. cerevisiae*. Substrates were different with respect to (i) ring substitution, (ii) side-chain constitution, and (iii) side-chain stereochemistry.

The effect of ring substitution in favoring decarboxylation is summarized in Table 1. When decarboxylation occurred, the reaction was complete in 24 hr, styrene was extracted with ether and analyzed by tlc and glc–ms by comparison with synthetic styrenes; by contrast, the substrate was recovered almost quantitatively after the same period in the first three cases of Table 1. A rationalization of substitution effects on benzene ring does not seem evident.

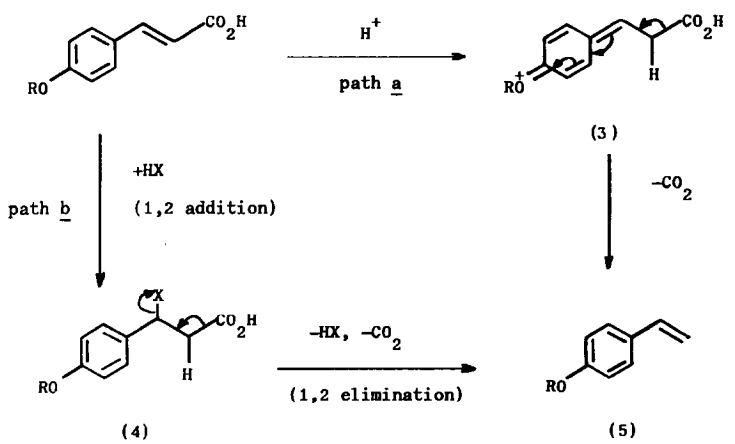
In regard to the reaction mechanism, two routes can be considered (Scheme 2).

Assuming path *b*, to account for the overall stereochemistry of the process, a *syn*-1,2-addition (Michael type) must be followed by an *anti*-1,2-decarboxylative elimination (or vice versa). This seems reasonable considering that a number of *syn*-Michael additions are known to occur *in vivo* (*14, 15*).

TABLE 1

DECARBOXYLATION OF RING-SUBSTITUTED CINNAMIC ACIDS

Acids	Decarboxylation
Cinnamic	Not
Caffeic	Not
3,4-Methylendioxycinnamic	Not
p-Coumaric	Yes
p-Methoxycinnamic	Yes
Ferulic	Yes
3,4-Dimethoxycinnamic	Yes



SCHEME 2

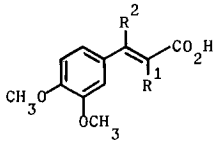
The hypothesis that β-hydroxyphenylpropionic acid was the product of the Michael addition was then examined. Since β-hydroxyphenylpropionic acids are unstable at pH 4–5 (usual fermentation pH value), giving rise to dehydration and decarboxylation products (*16, 17*), 3,4-dimethoxy-β-hydroxyphenylpropionic acid was synthesized and administered to resting cells of *S. cerevisiae* in a buffer medium at pH 8. The β-hydroxy acid was recovered unchanged, and no trace of styrene was found. By contrast, 3,4-dimethoxycinnamic acid is quantitatively decarboxylated in a blank experiment carried out under the same conditions. This finding, together with the fact that no β-hydroxyphenylpropionic acid was ever detected, even after short-term fermentation (1 hr), makes unlikely an hypothesis based on β-hydroxy acids as key intermediates in the decarboxylation reaction. Thus, the group X in Scheme 2 may be a nucleophilic group of an enzyme.

The decarboxylative elimination of an enzyme-bound intermediate is in agreement with results obtained on the *in vitro* decarboxylation of arylidenemalonic acids and β-substituted 3-phenylpropionic acids. A strong catalytic influence of thioacetic acid was observed for the decarboxylation of benzylidenemalonic acid derivatives and interpreted, on the basis of kinetic data, as due to the operation of an 1,2-addition followed by 1,2-elimination (*18*). This process seems a reasonable model for the enzymatic decarboxylation. The nucleophile-nucleofuge (*19*) of the enzyme active site could also be an hydroxy group in accordance with the easy decarboxylation of 3,4-dimethoxy-β-hydroxyphenylpropionic acid under mild acidic conditions reported above.

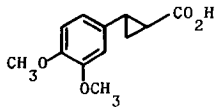
To investigate the effects of structural and stereochemical modifications of acrylic side chain, a number of 3,4-dimethoxycinnamic acids were tested for decarboxylation. From these experiments (Table 2) we could therefore conclude the following. (i) The configuration (*E*) of the double bond is necessary for the decarboxylation and such a double bond is not equated by a *trans*-cyclopropane ring. These results are likely a reflection of reaction requirements which appear essentially geometric in the first case and/or electronic in the latter. Substrate–enzyme binding could involve the carboxy group and a distal hydroxy or methoxy

TABLE 2

DECARBOXYLATION OF SIDE-CHAIN-SUBSTITUTED CINNAMIC ACIDS



(7)



(8)

a) R^1=CH_3 R^2=H

b) R^1=H R^2=CH_3

c) R^1=CH_2CH_3 R^2=H

ACIDS	DECARBOXYLATION
(Z)-3 ,4 -dimethoxycinnamic (6)	not
(E)-3 ,4 -dimethoxy-α-methylcinnamic (7a)	yes: (9)
(E)-3 ,4 -dimethoxy-β-methylcinnamic (7b)	" : (10)
(E)-3 ,4 -dimethoxy-α-ethylcinnamic (7c)	not
2-(3',4'-dimethoxyphenyl)-cyclopropanecarboxylic (8)	"

group in the benzene ring of the cinnamic acid: the distance between the two points of attachment of the substrate to the enzyme site would be optimum for the (*E*) configuration, but not for the (*Z*) one. Analogous geometric requirements have been invoked to explain the substrate and inhibitor specificity of some amino acid decarboxylases ((*20*) and references cited therein). The enzymatic specificity with respect to the double bond configuration might also be due to a conformational effect at the transition state level of the decarboxylation step (*21*). It is possible that the carboxy group of (3) or (4) attain an orientation perpendicular to the nodal π plane of forming styrenes (5) (*21*). Because of this stereoelectronic requirement a rotation around the C(2)–C(3) σ bond just after proton addition to C(2) must occur. Assuming an enzyme-controlled proton addition to the same face in both the (*E*)- and (*Z*)-cinnamic acids, the subsequent rotation would be in opposite directions for the (*E*) isomer (allowed rotation) and for the (*Z*) isomer (forbidden rotation). (ii) A threshold of steric hindrance seems to exist, in that a methyl group in the α or β position allows the reaction to occur, while an α-ethyl does not. (iii) A methyl group at the α position in the side chain does not affect the overall stereochemistry of the decarboxylation; this fact could provide a useful method for synthetizing (*Z*)-propenylphenols.

EXPERIMENTAL

Thin-layer chromatography was carried out on plates coated with Merck silica

gel GF_{254}, using benzene : ethyl formiate : formic acid (5 : 4 : 1) as eluent, unless otherwise stated. Merck silica gel (70–325 mesh) was used for column chromatography. Analytical glc was carried out on a Carlo Erba Fractovap 2400 V instrument with nitrogen as carrier gas (1.5 kg/cm^2) and glass columns containing neopentyl glicol succinate (LAC 767) (3%, w/w) on a silanized Chromosorb W (80–100 mesh). Mass spectra were determined with an LKB 9000 single focusing gas chromatograph–mass spectrometer fitted with a 3 × 4-mm glass column, packed as for analytical glc. Infrared spectra were recorded on a Perkin–Elmer 257 spectrometer, and nmr spectra on a 60-MHz Varian NV 14 spectrometer (tetramethylsilane as internal standard and deuterochloroform as solvent, unless otherwise stated). All compounds gave satisfactory elemental analyses. Samples of cinnamic, *p*-coumaric, *p*-methoxycinnamic, caffeic, ferulic, and 3,4-dimethoxycinnamic acids were from Merck–Schuchardt.

(*a*) *Preparation of Cinnamic Acids*

(E)-3,4-methylenedioxycinnamic acid. It was prepared according to Gill *et al.* (*22*). nmr (δ, DMSO): 7.52 (d, 1H, J 16 Hz, H_β), 7.4–6.8 (m, 3H, arom), 6.35 (d, 1H, J 16 Hz, H_α), 6.06 (s, 2H, O–CH_2–O).

(Z)-3,4-dimethoxycinnamic acid (*6*). 3,4-Dimethoxyphenylpropiolic acid (*23*) (206 mg) in methanol (25 ml) was hydrogenated using palladium-on-barium sulfate (5%, 41 mg) and freshly distilled quinoline (80 μl). After the consumption of one equivalent of hydrogen (20 min), the catalyst was removed by filtration and methanol evaporated. The residue was treated with water–HCl (10%) and extracted with ethyl acetate; usual workup gave pure (*Z*)-3,4-dimethoxycinnamic acid (146 mg). nmr (δ, DMSO): 7.7–6.8 (m, 3H, arom), 7.1 (d, 1H, J 13 Hz, H_β), 5.8 (d, 1H, J 13 Hz, H_α), 3.76 and 3.74 (2s, 3H each, 2 OCH_3).

β-(3,4-Dimethoxyphenyl)-β-hydroxypropionic acid (*5*). The Reformatzsky reaction on veratraldehyde according to Schmitt (*25*) afforded crude ethyl β-(3,4-dimethoxyphenyl)-β-hydroxypropionate. Distillation of the ester under reduced pressure (10^{-3} mm Hg) led to extensive dehydration, so the crude ester was directly saponified, as reported by Noyce for the corresponding 4-methoxypropionic acid (*17*). Ir (nujol): 3460 cm^{-1} ν O–H, 1705 cm^{-1} ν COOH sat. nmr (δ): 7.1–6.75 (m, 3H, arom), 6.34 (broad s, 1H, O*H*), 5.09 (m, X part of ABX system, 1H, H_β), 3.85 (s, 6H, 2 OCH_3), 2.77 (m, AB part of ABX system, 2H, CH_2).

(E)-3,4-Dimethoxy-α-methylcinnamic acid (*7a*). It was prepared by a Claisen condensation on veratraldehyde (*24*). nmr (δ): 7.86 (q, 1H, J 1 Hz, H_β), 7.3–6.8 (m, 3H, arom), 3.94 (s, 6H, 2 OCH_3), 2.21 (d, 3H, J 1 Hz, CH_3).

(E)-3,4-Dimethoxy-β-methylcinnamic acid (*7b*). It was prepared by Reformatzsky reaction on 3,4-dimethoxyacetophenone, according to Schmitt (*25*). nmr (δ): 7.4–6.75 (m, 3H, arom), 6.18 (q, 1H, J 1 Hz, H_α), 3.92 (s, 6H, 2 OCH_3) 2.6 (d, 3H, J 1 Hz, CH_3).

(E)-3,4-Dimethoxy-α-ethylcinnamic acid (*7c*). It was prepared according to the procedure adopted by Gensler for the synthesis of the corresponding methylendioxycinnamic acid (malonic condensation) (*26*) (yield 30%). mp = 122–123°C.

nmr (δ): 7.71 (broad s, 1H, H_β), 7.3–6.8 (m, 4H, 3 arom and COO*H*), 3.88 (s, 6H, 2 OCH_3), 2.63 (q, 2H, J 7 Hz, CH_2–CH_3), 1.22 (t, 3H, J 7 Hz, CH_2–CH_3).

2-(3',4'-Dimethoxyphenyl)-cyclopropane carboxylic acid (**8**). In a flask containing active zinc–copper couple (freshly prepared as reported by LeGoff (*27*)) ether (40 ml) and diiodomethane (2–3 ml) were added. A mixture of ethyl 3,4-dimethoxycinnamate (6 g) and diiodomethane (10 ml) was added dropwise, under stirring, to the exotermic reaction (2 hr). The reaction mixture was stirred at reflux for 72 hr. The ether solution was then slowly decanted into a mixture of ice–HCl, 1 *N*, separated, and washed with water (3 × 20 ml). After evaporation of the solvent, the residue was treated, as indicated above, with a new preparation of zinc–copper couple for 48 hr. The reaction mixture, after workup, was then treated with ozone at −10°C in carbon tetrachloride (10 ml). The ozonide was decomposed with dilute sodium hydroxide solution (10%, 10 ml) and the organic layer was dried and evaporated. The residual oil was hydrolized in boiling water–ethanol (1 : 1) for 3 hr. The alkaline solution was extracted with ether to remove veratraldehyde; after neutralization with HCl, extraction with ether and usual workup gave pure 2(3',4'-dimethoxyphenyl)cyclopropane carboxylic acid, which was crystallized from carbon tetrachloride (1.155 g). Ir (CCl_4): 3010 cm^{-1} ν C–H cyclopropane, 1695 cm^{-1} ν COOH. nmr (δ): 7.1–6.5 (m, 3H, arom), 3.82 (s, 6H, 2 OCH_3), 2.5 (m, 1H, H_β), 1.8 (m, 1H, H_α), 1.4 (m, 2H, CH_2).

(E)-3,4-Dimethoxy-[α-^{2}H]-cinnamic acid (**3**). It was prepared by Hofmann decomposition of α-trimethylammonio-3,4-dimethoxyphenylalanine in deuterated water, as reported by Manitto *et al.* (*28*). The percentage of deuteration was 86 ± 3% by nmr and ms spectra analysis.

(*b*) *Preparation of Styrenes*

4-Hydroxystyrene (**2a**) *and 3-methoxy-4-hydroxystyrene* (**2c**). They were prepared through decarboxylation of 4-hydroxy- and 3-methoxy,4-hydroxycinnamic acid, respectively (1 g), in vacuum (236–240°C at 1 mm Hg) with anhydrous sodium carbonate (0.5 g). (**2a**) glc–ms (nPGS 3%, 150° C, T_r = 10)(*m*/e,I%): 121(M^+ + 1,13), 120(M^+,100), 119(23), 91(37), 65(20). (**2c**) glc–ms (nPGS 3%, 150° C, T_r = 6.5): 150(M^+,100), 135(84), 107(60), 91(38), 77(40).

4-Methoxystyrene (**2b**) *and 3,4-dimethoxystyrene* (**2d**). They were prepared by methylation (methyliodure, potassium hydroxide) of the corresponding 4-hydroxystyrenes. (**2b**) glc–ms (nPGS 3%, 150°C, T_r = 1.5): 135(M^+ + 1,13), 134(M^+,100), 119(47), 91(59), 85(47), 83(72), 77(14), 65(35). (**2d**) glc–ms (nPGS 3%, 150°C, T_r = 4.6): 164(M^+,100), 149(55), 121(27), 103(38), 91(50), 78(27), 77(47).

(Z)-3,4-Dimethoxy-β-methylstyrene (**9**). It was prepared by alkaline isomerization (potassium hydroxide in isoamilic alcohol) of methyleugenol. glc–ms (nPGS 3%, 150°C, T_r = 6): 178(M^+,100), 163(47), 147(16), 135(13), 115(13), 107(45), 103(32), 91(42), 79(16), 77(21), 65(16).

3,4-Dimethoxy-α-methylstyrene (**10**). It was prepared through Grignard reaction on 3,4-dimethoxyacetophenone. glc–ms (nPGS 3% 150°C, T_r = 5.9): 178(M^+,100), 163(46), 135(25), 115(12), 107(38), 103(20), 95(40), 91(57), 79(29),

77(34), 65(25). All above compounds gave nmr spectra in accord with their structure.

(E)-3,4-Dimethoxy-[β-²H]-styrene (*4*). It was prepared, according to the method of Yoshino (*13*) for preparation of [β-²H]styrene, by Grignard reaction on (*E*)-3,4-dimethoxy-β-bromostyrene (this compound was prepared according to Trumbull for preparation of (*E*)-4-methoxy-β-bromostyrene (*29*)). nmr(δ): 7.05–6.8 (m, 3H, arom), 6.66 (broad d, 1H, J_{trans} 17.5 Hz, H_α), 5.58 (d, 1H, J_{trans} 17.5 Hz, H_β). glc–ms (nPGS 3%, 150°C, T_r = 4.6): 165(M^+,100), 164(30), 150(45), 122(25), 104(27), 92(38), 91(15), 79(24), 78(27), 77(20).

(*c*) *Fermentations*

Maintenance, growth, and fermentation conditions. Saccharomyces cerevisiae (MWC_{28}), kindly supplied by Dr. G. Albagnac (INRA de Dijon), was maintained on agar malt slants. Fermentations were started by inoculating cells into 750-ml cotton-plugged Erlenmeyer flasks with 150 ml of a medium containing 10% glucose, 0.7% yeast nitrogen base (Difco), 0.13% NaH_2PO_4. The flasks were incubated on alternative shaker at 27°C. After 24 hr the cells were recovered by centrifugation, washed twice with sterile distilled water, and resuspended in 150 ml of water to obtain a turbidity corresponding to 0.3 mg of N/ml. The cells' suspension was performed in phosphate buffer, 0.1 *M* (pH 8), for the transformation experiments with β-(3,4-dimethoxyphenyl)-β-hydroxypropionic acid. The substrates to be tested were then supplemented at a concentration of 0.06%. After 1, 4, 24, and 120 hr of incubation (aerobic conditions) the reaction mixtures were centrifuged at 5000 rpm and the supernatant liquid was then extracted with ether (3 × 50 ml).

Analysis of fermentation products. The extraction residue was analyzed by tlc and glc–ms (nPGS 3%, 150°C) by comparison with authentic commercial or synthetic samples. When no decarboxylation was observed, the almost complete recovery of starting acid was performed by extraction of supernatant liquid with ethyl acetate. The optimum fermentation time (24 hr, decarboxylation yield 80%) was determined by a gas chromatographic method (internal standard eugenol on nPGS 3% 150°C). In the experiment on deuterated material the decarboxylation product was recovered from three fermentation samples by purification of ethereal extract on silica gel column (benzene-light petroleum (bp 40–70°C) 1 : 1). The percentage of deuteration was 75 ± 3 by glc–ms analysis and deuterium position was confirmed as in (*Z*)-3,4-dimethoxy-[β-²H]styrene by nmr analysis. nmr (δ): 7.2–6.8 (m, 3H, arom), 6.64 (broad d, 1H, J_{cis} 10.5 Hz H_α), 5.1 (d, 1H, J_{cis} 10.5 Hz, H_β), 3.85 and 3.88 (2s, 3H each, 2 OCH_3). glc–ms (nPGS 3%, 150°C, T_r = 4.6): 165(M^+,100), 164(25), 150(41), 122(25), 104(21), 92(34), 91(17), 79(25), 78(27), 77(25).

REFERENCES

1. M. LUCKNER, "Secondary Metabolism in Plants and Animals," pp. 344–365. Chapman and Hall, London, 1972.

2. B. J. FINKLE, J. C. LEWIS, J. W. CORSE, AND R. E. LUNDIN, *J. Biol. Chem.* **237**(9), 2926 (1962).
3. R. J. PARRY, *Proc. Nat. Acad. Sci. USA* **72**(5), 1681 (1975).
4. S. R. INDAHL AND R. R. SCHELINE, *Appl. Microbiol.* **16**(4), 667 (1968).
5. G. C. WHITING AND J. G. CARR, *Nature (London)* **184**, 1427 (1959).
6. H. G. BAYNE, B. J. FINKLE, AND R. E. LUNDIN, *J. Gen. Microbiol.* **95**, 188 (1976).
7. A. D. WEBB AND C. J. MULLER, *Advan. Appl. Microbiol.* **15**, 75 (1972).
8. K. NISHIMURA AND M. MASUDA, *J. Food Sci.* **36**, 819 (1971).
9. R. F. LINDSAY AND F. G. PRIEST, *J. Appl. Bacteriol.* **39**, 181 (1975).
10. R. D. STEINKE AND M. C. PAULSON, *Agr. Food Chem.* **12**(4), 381 (1964).
11. G. ALBAGNAC, *Ann. Technol. Agr.* **24**(2), 133 (1975).
12. P. MANITTO, P. GRAMATICA, AND B. M. RANZI, *J. Chem. Soc. Chem. Comm.*, 442 (1975) (preliminary report).
13. T. YOSHINO, Y. MANABE, AND Y. KIKUCHI, *J. Amer. Chem. Soc.* **86**, 4670 (1964).
14. G. W. KIRBY, G. O'LOUGHLIN, AND D. J. ROBINS, *J. Chem. Soc. Chem. Comm.*, 402 (1975).
15. K. R. HANSON AND I. A. ROSE, *Accounts Chem. Res.* **8**, 1 (1975).
16. D. S. NOYCE, G. L. WOO, AND P. A. KING, *J. Org. Chem.* **26**, 632 (1961).
17. D. S. NOYCE, P. A. KING, C. A. LANE, AND W. L. REED, *J. Amer. Chem. Soc.* **84**, 1638 (1962).
18. E. J. COREY AND G. FRAENKEL, *J. Amer. Chem. Soc.* **75**, 1168 (1953).
19. C. J. M. STIRLING, *Accounts Chem. Res.* **12**, 198 (1979).
20. M. H. O'LEARY AND G. J. PIAZZA, *J. Amer. Chem. Soc.* **100**, 632 (1978).
21. M. H. O'LEARY, "Bioorganic Chemistry" (E. E. vanTamelen, Ed.), Vol. 1, pp. 259–275. Academic Press, New York/London, 1977.
22. G. S. GILL, H. K. GAHLAR, N. K. RALHAN, AND K. S. NARANG, *Indian J. Chem.* **3**(7), 323 (1965).
23. F. G. BADDAR, H. A. FAHIM, AND M. A. GALABY, *J. Chem. Soc.*, 465 (1955).
24. W. S. IDE AND J. S. BUCK, *J. Amer. Chem. Soc.* **62**, 425 (1940).
25. J. SCHMITT, M. SUQUET, P. COMOY, J. BOITARD, G. CALLET, T. CLIM, AND J. LE MEUR, *Bull. Chim. Soc. Fr.*, 953 (1966).
26. W. J. GENSLER AND E. BERMAN, *J. Amer. Chem. Soc.* **80**, 4949 (1958).
27. E. LEGOFF, *J. Org. Chem.* **29**, 2048 (1964).
28. P. MANITTO, D. MONTI, P. GRAMATICA, AND E. SABBIONI, *J. Chem. Soc. Chem. Comm.*, 563 (1973).
29. E. R. TRUMBULL, R. T. FINN, K. M. IBNE-RASA, AND C. K. SAUERS, *J. Amer. Chem. Soc.*, **84**, 2339 (1962).