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Further studies on the biosynthesis of the avermectins

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SUMMARY

The biosynthesis of avermectins was studied further in *Streptomyces avermitilis* MA5502 by feeding experiments with labeled precursors. ^{13}C-NMR analysis of the compounds biosynthesized from [2-^{13}C]acetate, [1,2-$^{13}C_2$]acetate, [3-^{13}C]propionate and [2,3-$^{13}C_2$]propionate confirmed that the aglycone of avermectins is made from seven intact acetate and five propionate units. Feeding experiments with [1-^{13}C]2-methylbutyrate and [1-^{13}C]isobutyrate have shown that 2-methylbutyrate and isobutyrate are immediate precursors of the starter units of the polyketide chains of avermectin 'a' and 'b' components, respectively. The ^{3}H/^{14}C double-labeling experiments suggest that the two oleandrose moieties are derived from glucose.

INTRODUCTION

The avermectins (Fig. 1) are a new family of naturally occurring macrolides produced by *Streptomyces avermitilis* [3]. They possess potent anthelmintic as well as insecticidal activities [7]. In addition to the present usage in the animal health field, these compounds have potential utility in agriculture and in human parasitic infections. Although their structures are closely related to the macrolide antibiotics, they possess no significant antibiotic activity. Mode of action studies [5,6] have suggested that avermectin B_1 stimulates chloride ion conductance, resulting in hyperpolarization of the membrane.

Structure elucidation [1,8], based on chemical and spectroscopic means, showed that the avermectins represent a novel combination of several structural features. The 16-membered macrocyclic ring of the aglycone of the avermectins includes a hexahydrobenzofuran ring system and two 6-membered spiroketal rings. The sugar component consists of two molecules of L-oleandrose. The eight avermectin components, Ala through B2b carry various substituents at the cyclohexenyl and spiroketal structure units, i.e., hydroxy or methoxy groups at C-5, an axial-α-hydroxy group at C-23 or a C-22, C-23-double bond, and a *sec*-butyl or isopropyl groups attached to C-25.

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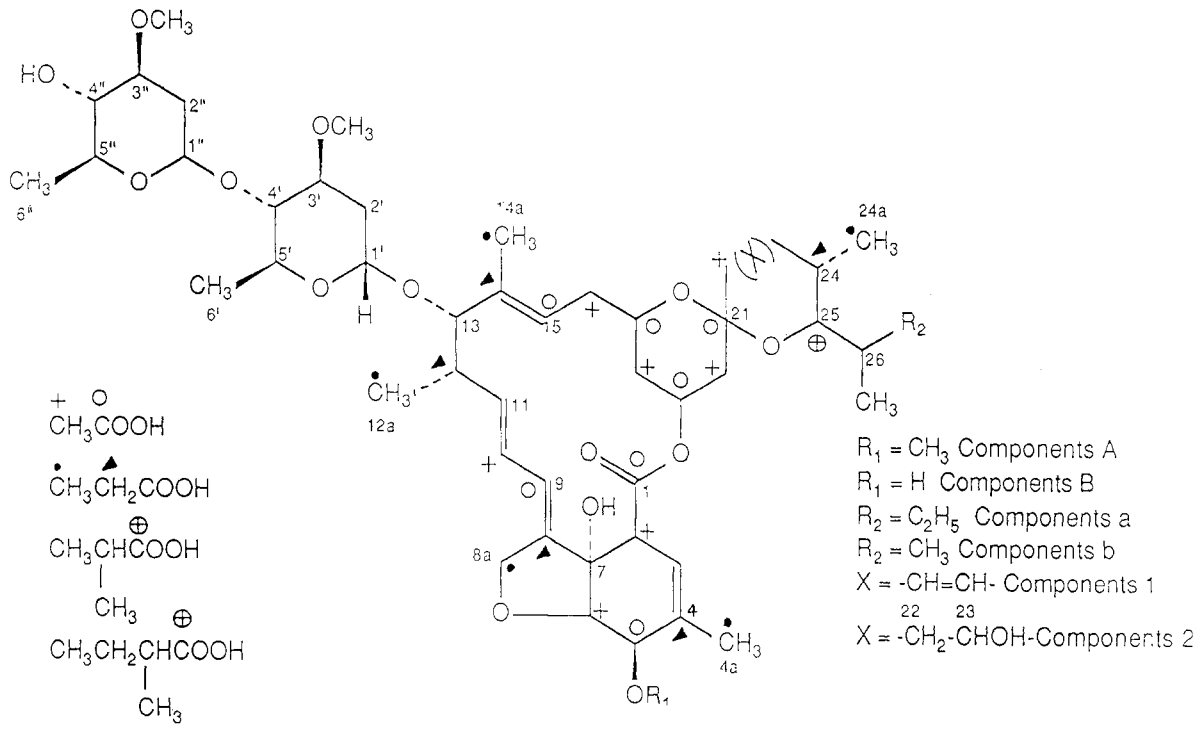

Fig. 1. Labeling pattern of the aglycone moiety of avermectins.

In this communication, we report results* which establish the biosynthetic origin of avermectins.

EXPERIMENTAL SECTION

General methods and materials

^{13}C-NMR spectra were recorded on Varian SC-300 and Varian XL-200 nuclear magnetic resonance spectrometers operating at 75.6 Hz and 50.4 Hz, respectively. Chemical shifts are given in parts per million (ppm) relative to the C_6H_6 resonance at o_c 127.7 ppm. Radioactivity measurements were by liquid scintillation counting in a Packard 3330 spectrometer using a commercial Scinti Verse I (Fisher Scientific Company) solution. Counting efficiencies were determined using a commercial 'Oxi-Test' (Radiomatic Instruments and Chemical Co.) ^{3}H or ^{14}C internal standard. Radiolabeled compounds were purchased from Amersham or New England Nuclear and used without further purification. ^{13}C-Labeled compounds were obtained from Merck Sharp and Dohme. In all experiments with ^{13}C-labeled precursors, a small amount of the ^{14}C-labeled compound was fed along with the stable isotope-labeled sample in order to allow determination of the approximate average enrichment from the specific radioactivity of the product. ^{13}C-Labeled precursors containing radioactive internal standard in distilled water were sterilized by passage through a sterile 0.22 μm Millipore filter and added in three portions to the fermentation at 60, 84, 108 h to achieve the following final concentrations: 2-methylbutyrate 1 mg/ml, isobutyrate 0.8 mg/ml, propionate 1 mg/ml and acetate 1.5 mg/ml. In the double-labeled glucose experiments isolated avermectin A2a was subjected to methanolysis as described by Albers-Schonberg et al. [1] to yield methyl oleandroside. The integrations of the spectra for both the ^{13}C-enriched material and the natural abundance material were first normalized using the signal in-

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tensity of C-3″ as 1.1. The relative ^{13}C abundance was then calculated according to the following equation:

$$^{13}C \text{ Relative abundance} = \frac{\text{Normalized integration in the enriched spectrum}}{\text{Normalized integration in the natural abundance spectrum}} \times 1.1$$

Culture conditions

Vegetative inoculum stored in liquid nitrogen was used as the culture source. Inocula were incubated overnight in 250-ml baffled flasks containing 20 ml of seed medium on a rotary shaker. Seed medium contained (in grams per 1000 ml of water) the following: Dextrose, 20.0; Hycase, S.F., 20.0; Yeast extract, 20.0; KNO_3, 2.0; $FeSO_4 \cdot 7H_2O$, 0.025; $CaCl_2 \cdot 2H_2O$, 0.02; $ZnSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot H_2O$, 0.005; NaCl, 0.5. One milliliter of the culture broth thus obtained was transferred into a 250-ml unbaffled Erlenmeyer flask containing 20 ml of a modified medium B as described by Burg et al. [3]. Incubation was carried out on the rotary shaker at 28°C for 8 days.

Isolation of avermectins

For the isolation of avermectins, broth pH was adjusted first to 9.0 by addition of sodium hydroxide solution. The mycelium and broth were then separated by centrifugation at 3000 rpm and the mycelium was extracted with methanol. The combined organic extracts were concentrated to remove methanol and the dichloromethane resultant solution was extracted with dichloromethane. The extracts were filtered and dried over sodium sulfate. Evaporation of the solvent gave a dark colored residue, which was redissolved in a small amount of dichloromethane and adsorbed on a column of silica gel (Mallinckrodt). The column was eluted with 15% isopropyl alcohol in dichloromethane. The final purification was carried out by preparative HPLC on a Dupont Zorbax C-18 column (21.2 mm × 25 cm) at room temperature using MeOH/H_2O (85:15) as the mobile phase. Approximately 15 mg of ^{13}C-labeled avermectin was purified for ^{13}C-NMR.

RESULTS

The biosynthesis of avermectin was studied in *S. avermitilis* MA5502 which was grown in a modified production medium B. On the basis of time course studies, the labeled precursors were dispensed to the cultures at 48, 72 and 96 h after inoculation, and the cultivation was continued for an additional 96 h. Avermectins were isolated as described in the experimental section.

A complete ^{13}C-NMR analysis was carried out in order to provide the signal assignments essential for this study. An unequivocal assignment of every signal in the spectrum rests on the characteristic chemical shifts, multiplicities, single-frequency decoupling, comparison with several derivatives, and analysis of one-bond carbon–carbon couplings of pairs of carbon atoms.

The structure of the avermectins strongly suggests that their formation is by the polyketide pathway. In analogy to the formation of most other macrolide natural products one might expect avermectins to be of a mixed acetate/propionate origin, e.g., the avermectin Bla molecule would be made up of eight acetate (A) and six propionate units (P) in a sequence (Me)A-P-P-A-A-A-A-P-P-A-P-A-P-A (COOH). As an alternative, the *sec*-butyl moiety (isopropyl moiety for 'b' components) attached to C-25 might have resulted from the use of 2-methylbutyryl (isobutyryl for 'b' components) unit in the form of 2-methylbutyryl-CoA (isobutyryl CoA for 'b' components) as the chain starter unit. On the other hand, the methyl branches might arise entirely by C-methylation of acetate units.

According to previous experiments with [1-^{13}C]acetate and [1-^{13}C]propionate carried out by Cane et al. [4] using *S. avermitilis* strain MA5192, carbon atoms 1, 5, 9, 15, 17, 19 and 21 arise from C-1 of acetate and carbon atoms 3, 7, 11, 13 and 23 from C-1 of propionate. We carried out experiments with [2-^{13}C]acetate and [3-^{13}C]propionate to further explore the modes of their incorporation.

Table 1

Carbon No.	Chemical shift δ C (ppm)	Relative ^{13}C abundance in avermectin B1a biosynthesized from			$^{1}J_{C-C}$ in avermectin B1a from	
		[2-^{13}C]-acetate	[3-^{13}C]-propionate	[1-^{13}C]-2-methylbutyrate	[1,2-$^{13}C_2$]-acetate	[2,3-$^{13}C_2$]-propionate
C-1	173.4	1.4	1.0	1.1	59.3 Hz	
C-2	45.8	3.2	1.2	0.9	59.3 Hz	
C-3	117.8	1.8	1.4	6.7		
C-4	135.4	3.0	1.2	1.3		43.4 Hz
C-4a	19.8	2.2	5.8	1.2		43.4 Hz
C-5	67.6	1.0	1.3	0.8	40.7 Hz	
C-6	79.1	3.8	1.2	1.0	40.7 Hz	
C-7	80.4	1.6	1.4	7.7		
C-8	141.0	2.8	1.3	1.2		43.2 Hz
C-8a	68.3	2.4	6.2	1.0		43.2 Hz
C-9	120.0	1.1	1.0	1.2	55.9 Hz	
C-10	125.2	3.2	1.3	0.9	55.9 Hz	
C-11	137.2	1.8	1.5	6.4		
C-12	39.8	2.6	1.5	1.0		35.5 Hz
C-12a	20.1	2.4	6.8	1.1		35.5 Hz
C-13	81.6	1.2	1.4	5.9		
C-14	138.3	2.9	0.9	0.9		44.0 Hz
C-14a	15.0	2.3	6.6	1.1		44.0 Hz
C-15	118.8	1.1	1.2	1.0	43.7 Hz	
C-16	34.5	3.0	1.1	1.1	43.7 Hz	
C-17	68.3	1.2	1.1	1.3	36.3 Hz	
C-18	36.9	4.4	1.3	1.0	36.5 Hz	
C-19	68.3	1.2	1.1	1.3	26.3 Hz	
C-20	41.0	3.8	1.3	0.9	36.3 Hz	
C-21	96.0	1.3	0.9	1.2	54.9 Hz	
C-22	128.7[a]	3.7	1.3	1.0	54.9 Hz	
C-23	135.2[a]	1.8	1.5	5.6		
C-24	30.4	2.7	1.7	0.9		35.2 Hz
C-24a	16.3	2.8	6.7	1.2		35.2 Hz
C-25	74.8	2.2	1.0	24.5		
C-26	35.1	1.3	1.1	0.8		
C-26a	12.9	1.3	1.0	0.9		
C-27	27.4	1.4	1.0	1.1		
C-28	11.9	1.3	1.0	0.9		
C-1′	95.0	1.2	1.0	0.9		
C-1″	98.8	1.1	1.0	1.0		
C-2′	34.4	1.3	1.0	1.0		
C-2″	34.4	1.2	1.0	1.0		
C-3′	79.3	1.2	1.0	1.0		
C-3″	78.1	1.1	1.1	1.1		
C-4′	80.3	1.1	1.1	1.1		
C-4″	75.8	1.1	1.1	1.0		
C-5′	67.1	1.1	1.3	0.8		
C-5″	68.0	1.0	1.1	1.0		
C-6′	18.3	1.0	1.1	1.0		
C-6″	17.6	1.0	1.1	1.0		
3′,3″-O-CH_3	55.7	1.7	1.2	1.3		

[a] These values may be reversed. The assignments listed are the ones compatible with biosynthetic theory.

The ^{13}C-distributions in avermectin Bla are shown in Table 1. In the experiment with [3-^{13}C]propionate five methyl carbons (4a, 8a, 12a, 14a and 24a) were labeled. Subsequent incorporation of [2,3-$^{13}C_2$]propionate gave rise to a set of five pairs of enhanced and coupled doublets. The labeling patterns from [2-^{13}C]acetate are not as clear-cut due to metabolic scrambling of the label. Nevertheless, it is evident that carbon atoms 2, 6, 10, 16, 18, 20 and 22 of avermectin Bla are labeled by C-2 of acetate; however, it is not clear whether this represents a direct conversion of acetate into those carbons or some indirect incorporation of the label. The proton noise-decoupled (p.n.d.) ^{13}C-NMR spectrum of avermectin Bla obtained from [1,2-$^{13}C_2$]acetate clearly shows that the following pairs of carbons are coupled and therefore came from a single molecule of doubly labeled acetate: C-1 and C-2, C-5 and C-6, C-9 and C-10, C-15 and C-16, C-17 and C-18, C-19 and C-20, C-21 and C-22.

The results thus establish the origin of the aglycone of the avermectins except for the moieties comprised of C-25 and the attached *sec*-butyl side-chain of the 'a' components and the isopropyl side-chain of the 'b' components. Feeding experiments with [U-^{14}C]isoleucine and [U-^{14}C]valine carried out by Albers-Schonberg et al. [2] gave high specific incorporation into avermectin Bla and Blb, respectively, indicating that isoleucine and valine or their metabolic products might be the precursors of the starter units of the polyketide chains. To test this possibility further, we carried out preliminary experiments with [1-^{14}C]isobutyrate. The results are shown in Table 2. The efficient incorporation into avermectin Blb suggests that isobutyrate may be a more immediate precursor for C-25 side-chain of Blb than is valine. In order to obtain more conclusive data to support our polyketide pathway hypothesis, feeding experiments with [1-^{13}C]2-methylbutyrate and [1-^{13}C]isobutyrate were carried out and avermectins Bla and Blb were purified. The ^{13}C-NMR data are shown in Table 1. [1-^{13}C]2-Methylbutyrate results in the enhancement of the signals for carbons 3, 7, 11, 13, 23 and 25. The ^{13}C-enrichment of C-25 is 24%, indicating that the C-25 side-chain of 'a' components is derived from 2-methylbutyrate. The ^{13}C-enrichment of the other carbons is around 5–6%. Since 2-methylbutyrate can be metabolized to propionate by losing acetyl CoA, carbon 1 of 2-methylbutyrate would then be

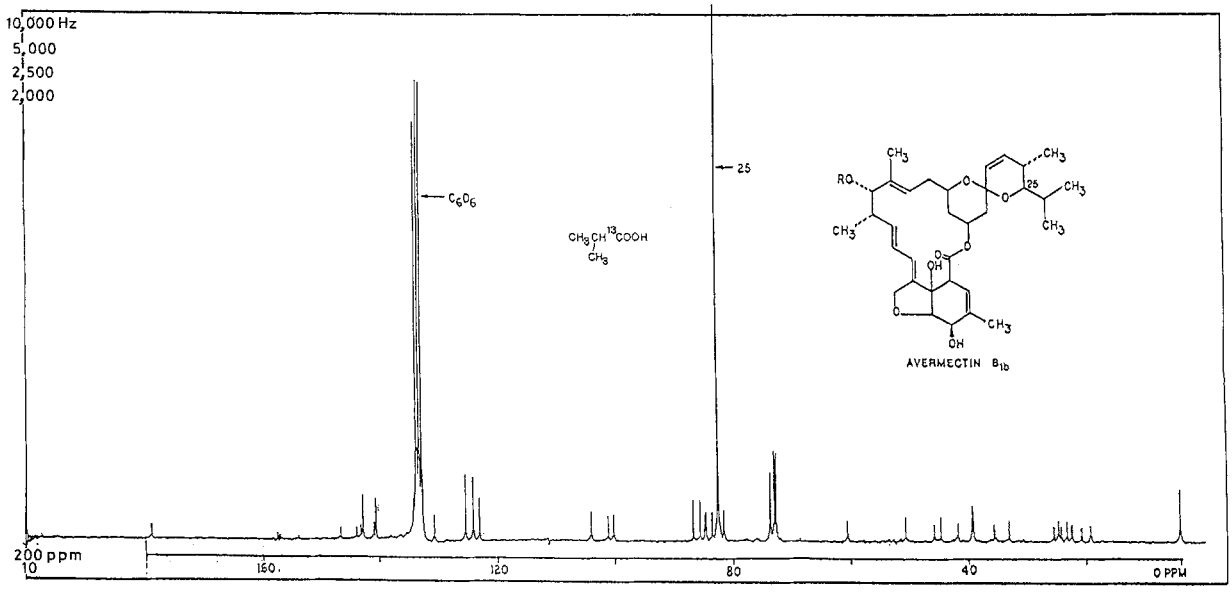

Fig. 2. Proton noise-decoupled ^{13}C-NMR spectrum of avermectin B_{1b} biosynthetically enriched with [^{13}C]isobutyrate.

Table 2

The specific incorporation of sodium isobutyrate as a function of the amount of sodium isobutyrate fed

Sodium isobutyrate fed		Avermectins formed			
Amount added (mmol/l)	Spec. radioact. (μCi/mmol)	Spec. radioact. (μCi/mmol) of		Spec. incorporation (%) into	
		Bla	Blb	Bla	Blb
1.1	11	0.17	0.57	1.5	5
2.2	6.36	0.41	0.69	6.4	10.9
3.3	3.62	0.71	0.82	4.7	22.7
4.4	2.58	0.27	0.77	10.5	29.8

labeled in carbon 1 of propionate. This gives an explanation for the observed enriched signals of carbon atoms 3, 7, 11, 13 and 23 of avermectin Bla. The p.n.d. ^{13}C-NMR spectrum (Fig. 2) due to [1-^{13}C]isobutyrate-derived avermectin Blb shows one highly enhanced signal at C-25, confirming that isobutyrate is the immediate precursor of the starter unit of 'b' components.

Evidence to support a direct conversion of glucose into the oleandrose moieties of avermectins comes from the double-labeling experiments. The results are shown in Table 3. [6-^{14}C, 6-^{3}H]Glucose ([^{3}H]/[^{14}C] 0.46) gave rise to avermectin A2a ([^{3}H]/[^{14}C] 0.10 = 21.7% ^{3}H-retention), reflecting the fact that much of the tritium is lost during metabolic breakdown of glucose. However, methyl oleandrose obtained by methanolysis of this avermectin has almost the same [^{3}H]/[^{14}C] ratio ([^{3}H]/[^{14}C] 0.46 = 100% ^{3}H-retention) as the precursor, suggesting that most of the radioactivity in the deoxy sugar moieties is due to a direct conversion of glucose into the oleandrose portion of the compound. Moreover, [6-^{14}C, 1-^{3}H]glucose ([^{3}H]/[^{14}C] 0.91) gave rise to avermectin A2a ([^{3}H]/[^{14}C] 0.14 = 15.4% ^{3}H-retention) and the methyl oleandroside obtained by the methanolysis contained tritium ([^{3}H]/[^{14}C] 0.67 = 74% ^{3}H-retention) at a substantially higher [^{3}H]/[^{14}C] ratio than A2a.

CONCLUSION

The ^{13}C-labeling studies reported here demonstrate quite clearly that the aglycone of avermectins is biosynthesized by the polyketide pathway in the manner indicated in Fig. 1. The aglycone is formed from, presumably, a single polyketide chain, starting at C-25 side-chain and ending at C-1. This polyketide chain thus contains seven acetates and five propionate units. In one of the propionate units, the methyl group (C-8a) has been oxidized to an alcohol. 2-Methyl-butyrate and isobutyrate are immedi-

Table 3

Incorporation of specifically labeled glucose into avermectin A2a and methyl oleandrose

Position of label in glucose fed	^{3}H/^{14}C of glucose	Avermectin A2a		Methyl oleandrose	
		^{3}H/^{14}C	^{3}H retention (%)	^{3}H/^{14}C	^{3}H retention (%)
6-^{14}C, 1-^{3}H	0.91	0.14	15.4	0.67	74
6-^{14}C, 6-^{3}H	0.46	0.10	21.7	0.46	100

ate precursors of the starter units of the polyketide chains of 'a' and 'b' components, respectively. The two oleandrose moieties of the avermectins are presumably of identical origin and both should be derived from glucose. The double-labeling experiments with glucose support this assumption and point to intact conversion of the carbon skeleton of glucose into that of oleandrose.

REFERENCES

1 Albers-Schonberg, G., B.H. Arison, J.C. Chabala, A.W. Douglas, P. Eskola, M.H. Fisher, A. Lusi, H. Mrozik, J.L. Smith and R.L. Tolman. 1981. Avermectins. Structure Determination. J. Am. Chem. Soc. 103: 4216–4221.

2 Albers-Schonberg, G., R.T. Goegelman, L. Kaplan, A. Kempf and J.B. Tunac. unpublished results.

3 Burg, R.W., B.M. Miller, E.E. Baker, J. Birnbaum, S.A. Curri, R. Hartman, Y.-L. Kong, R.L. Monaghan, G. Olson, I. Putter, J.-B. Tunac, H. Wallick, E.O. Stapley, R. Oiwa and S. Omura. 1979. Avermectins, new family of potent anthelmintic agents: Producing organism and fermentation. Antimicrob. Agents Chemother. 15: 361–367.

4 Cane, D.E., T.-C. Ziang, L. Kaplan, M.K. Nallin; M.D. Schulman, O.D. Hensens, A.W. Douglas and G. Albers-Schonberg. 1983. The biosynthetic origin of the carbon skeleton and oxygen atoms of the avermectins. J. Am. Chem. Soc. 105: 4110–4112.

5 Kass, I.S., C.C. Wang, J.P. Walrond and A.O.W. Stretton. 1980. Avermectin Bla, a new paralyzing anthelmintic that affects interneurons and inhibitory motor-neurons in Ascaris. Proc. Natl. Acad. Sci. U.S.A. 77: 6211–6215.

6 Pong, S.S., R. Dehaven and C.C. Wang. 1982. A comparative study of avermectin Bla and other modulators of the α-aminobutyric acid receptor chloride ion channel complex. J. Neuroscience 2: 966–971.

7 Putter, I., J.G. MacConnell, F.A. Preiser, S.S. Ristich and R.A. Dybas. 1981. Avermectins: novel insecticides, acaricides and nematicides from a soil microorganism. Experientia 37: 963–966.

8 Springer, J.L., B.H. Arison, J.M. Hirschfield and K. Hoogsteen. 1981. The absolute stereochemistry and conformation of avermectin B2a aglycone and avermectin Bla. J. Am. Chem. Soc. 103: 4221–4224.