Mechanism of Brevicomin Biosynthesis from (Z)-6-Nonen-2-one in a Bark Beetle[1]

Désirée Vanderwel*,[2] and A. Cameron Oehlschlager

Contribution from the Department of Chemistry, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6. *Received December 12, 1991*

Abstract: We have recently shown that (*Z*)-6-nonen-2-one serves in vivo as a precursor of the widely occurring bark beetle pheromone (+)-*exo*-brevicomin. The objective of this study was to determine the biochemical mechanism of this transformation. A novel application of stable isotope labeling techniques, involving the incorporation of the stable isotope ^{18}O from labeled water and oxygen, has now been used to determine the nature of the biosynthetic intermediate and the stereochemistry of the biochemical transformation. The mountain pine beetle (MPB), *Dendroctonus ponderosae* Hopkins, was used as the test insect. MPBs exposed to [^{18}O]oxygen gas produced *exo*-brevicomin that was enriched in two atoms of ^{18}O/molecule, indicating that both oxygens of *exo*-brevicomin were derived from molecular oxygen. These results indicated that the conversion of (*Z*)-6-nonen-2-one to (+)-*exo*-brevicomin proceeded through a keto epoxide intermediate and that the epoxide was not converted to a diol prior to the cyclization. Labeling studies with [^{18}O]oxygen gas and [^{18}O]water indicated that the conversion of the keto epoxide to (+)-*exo*-brevicomin in MPBs proceeded through both enantiomers of the keto epoxide derivative of (*Z*)-6-nonen-2-one, (6*S*,7*R*)-and (6*R*,7*S*)-6,7-epoxynonan-2-one. The route through the (6*S*,7*R*) keto epoxide was favored in males, while that through the (6*R*,7*S*) keto epoxide was favored in females.

The bicyclic acetal *exo*-brevicomin (**1**) is used as a pheromone by several species of economically important scolytid bark beetles.[3] The biosynthetic origin of this and several structurally related bicyclic and tricyclic acetals that function as pheromones has been the subject of considerable speculation.[4] Silverstein suggested that the biosynthetic pathways to the cyclic acetals may be similar to laboratory routes to these pheromones.[5] (*Z*)-6-Nonen-2-one (**2**), a laboratory precursor of **1**[6], was found together with **1** in the ash bark beetle *Leperisinus varius* by Francke, who speculated about the biogenic relationship between the two compounds.[7] We recently reported that **2** serves as a precursor of the pheromone in vivo.[8] Using stable isotope labeled **2**, we found that the bark beetles *Dendroctonus ponderosae* Hopkins (the mountain pine beetle, MPB) and *Dryocoetes confusus* Swaine (the western balsam bark beetle) converted this precursor to (+)-**1**.[8]

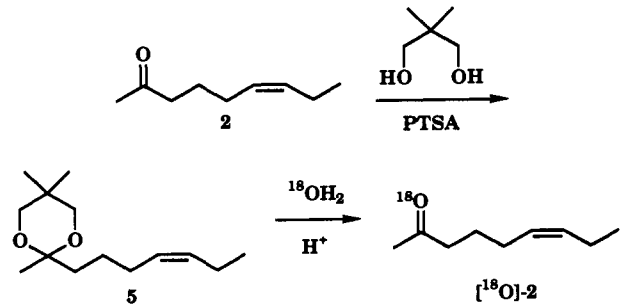
We now report the mechanism of the conversion of **2** to (+)-**1** in male MPBs. Although the simplest process for this conversion involves epoxidation and cyclization, it is possible to envision numerous discrete intermediates that could be involved (Figure 1). For example, an initially formed keto epoxide (**3**) could be acted upon by a hydrolase to produce several keto diols (**4**). The latter could either cyclize directly or be derivatized at one hydroxyl (as, for example, a phosphate) before conversion to **1**. The biosynthetic process utilized by MPBs, as well as the stereochemistry of the conversion, was determined by a novel application of stable isotope labeling techniques.

Results and Discussion

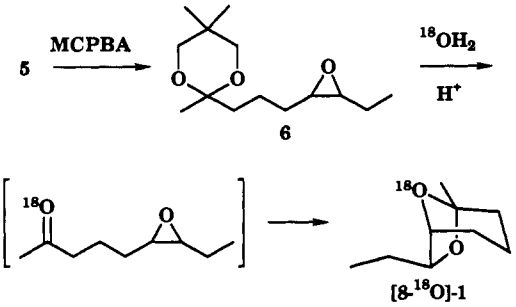
Syntheses of Labeled Compounds. The oxygen-18 label was introduced into **2** by acid-catalyzed hydrolysis of the 5,5-dimethyl-1,3-dioxane derivative (**5**) in the presence of [^{18}O]water (Scheme I). *exo*-Brevicomin (**1**) was ^{18}O-labeled according to Scheme II. Thus **6** was hydrolyzed by [^{18}O]water and then immediately cyclized to **1** by heating in the presence of acid. To prevent the loss of label, the intermediate keto epoxide was not isolated. As judged by GC/SIM/MS analysis, **1** prepared by this route was 96% enriched for ^{18}O. The MS(EI), ^1H NMR, and ^{13}C NMR spectra of **1** were in agreement with those previously published.[9]

The ^{18}O label in **1** was unambiguously determined to be in position 8 by ^{13}C NMR spectroscopy. The resonances of carbons singly bonded to ^{18}O exhibit an upfield shift of between 0.010 and 0.035 ppm compared to those bonded to ^{16}O.[10] This small difference is difficult to detect by comparison of the ^{13}C NMR spectra of labeled and unlabeled compounds. For this reason, synthetic unlabeled **1** and [^{18}O]-**1** were mixed in a ratio of 2:1 prior to

Scheme I. Synthetic Route to [2-^{18}O]-(*Z*)-6-Nonen-2-one ([^{18}O]-**2**)



Scheme II. Synthetic Route to [8-^{18}O]-*exo*-Brevicomin ([^{18}O]-**1**)



* To whom correspondence should be addressed.

(1) We would like to thank G. Owen for assisting with the mass spectral analyses; M. Tracey for assisting with the NMR analyses; D. R. Miller, G. Gries, and J. H. Borden for supplying the insects used in this study; R. W. Currie for assisting with the statistical analyses; B. M. Pinto for helpful discussions; and the Frank Allison Linville Institute, the Natural Science and Engineering Research Council of Canada, and Simon Fraser University for the support of this research.

(2) New address of D.V. is Department of Chemistry, University of Winnipeg, 515 Portage Ave., Winnipeg, MB, Canada R3B 2E9.

(3) Borden, J. H. In *Comprehensive Insect Physiology Biochemistry and Pharmacology*; Kerkut, G. A., Gilbert, L. I., Eds.; Pergamon Press: New York, 1985; Vol. 9, pp 257–285.

(4) Vanderwel, D.; Oehlschlager, A. C. In *Pheromone Biochemistry*; Prestwich, G. D., Blomquist, G. J., Eds.; Academic Press, Inc.: Orlando, FL, 1987; pp 175–215.

(5) Silverstein was cited in the following: White, R. A., Jr.; Agosin, M.; Franklin, R. T.; Webb, J. W. *Z. Angew. Entomol.* **1980**, *90*, 255–274.

(6) Rodin, J. O.; Reece, C. A.; Silverstein, R. M.; Brown, V. H.; Degraw, J. I. *J. Chem. Eng. Data* **1971**, *16*, 380–381.

0002-7863/92/1514-5081$03.00/0

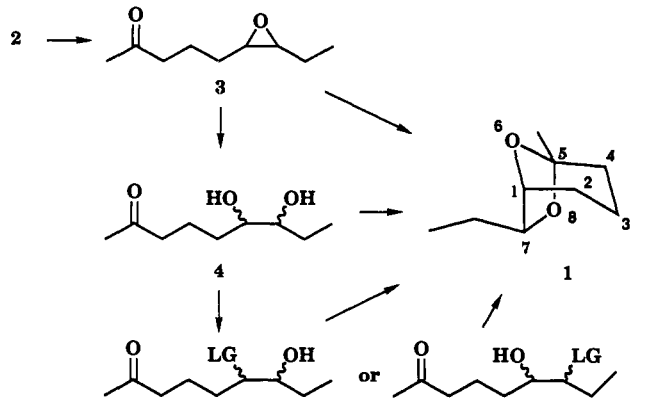


Figure 1. Hypothesized intermediates in the conversion of (*Z*)-6-nonen-2-one (**2**) to (+)-*exo*-brevicomin (**1**). The numbering system for **1** is also denoted. (LG denotes leaving group.)

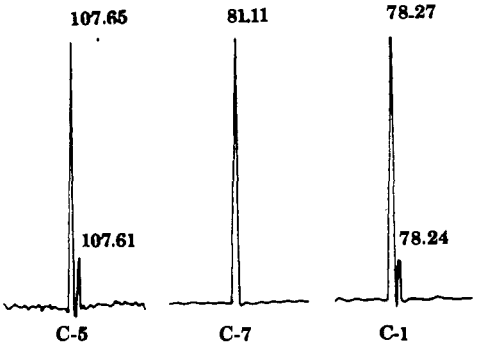


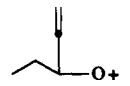
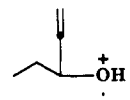
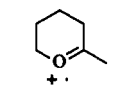
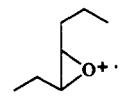
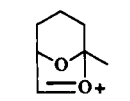
Figure 2. ^{13}C NMR resonances of C-1, C-5, and C-7 of unlabeled **1** and [^{18}O]-**1**, mixed in a 2:1 ratio (respectively).

examination of the ^{13}C NMR spectra (Figure 2). The signals due to C-1 and C-5 were shifted upfield by 0.03 and 0.04 ppm, respectively, in [^{18}O]-**1** compared to unlabeled [^{16}O]-**1**. However, the signal due to C-7 was not shifted in the [^{18}O]-**1**. This indicates that the ^{18}O was in position 8.

Comparison of MS (EI) Fragmentation Patterns of Unlabeled 1 and [8-^{18}O]-1. To determine the mechanism of conversion of **2** to **1** by MPBs it was required to determine the labeling patterns in **1** produced from different sources of oxygen-18. Since MPBs produce less than 1 μg of extractable **1** and we wished to be able to conduct the analyses on extracts of single insects, mass spectral analysis was the method of choice. In initial experiments we used the [8-^{18}O]-**1** prepared above to determine which fragments contained O-8. Comparisons of the mass spectra of [8-^{18}O]-**1** and unlabeled **1** are summarized in Tables I and II. In Table I the identity of the fragment ions as assigned by Gore et al.[9] is given with their expected *m/z* values in labeled and unlabeled **1**.

The usefulness of the major fragment ions for determination of the position of the ^{18}O label in **1** produced by MPBs was evaluated by comparison of their observed masses when produced from unlabeled **1** and [8-^{18}O]-**1** (Table II). The fragment ion pairs at (*m/z*) 85/87, 98/100, and 114/116 were judged to give the most incisive information. To maximize sensitivity by GC/SIM/MS, it was desirable to scan as few ion masses as possible. The fragment ion pair at 85/87 was eliminated from consideration since the fragment ion at m/z = 86 has a $(F + 1)^+$ ion at m/z = 87 which corresponds to roughly 10% of its intensity due to the natural abundance of heavy isotopes and interferes significantly

Table I. Expected Major Fragment Ions by MS (EI) of Unlabeled **1** and [8-^{18}O]-**1**[a]

Fragment	Expected m/z of Fragments: 1	[8-^{18}O]-1	[6-^{18}O]-1
	43	45	43
	85	85	87
	86	86	88
	98	100	98
	114	114	116
	127	129	129

[a] Given ion structures are hypothetical.

Table II. Comparison of Observed Relative Abundances of Major MS (EI) Fragments in Unlabeled **1** and [8-^{18}O]-**1**

fragments $F^+/(F + 2)^+$	ratio of relative abundances of fragments: unlabeled **1**	[8-^{18}O]-**1**
43/45	100.0/nd[a]	68.1/100.0
114/116	100.0/nd	100.0/21.8
86/88	100.0/nd	83.0/100.0
85/87	100.0/14.8	100.0/16.6
98/100	100.0/3.9	24.2/100.0
127/129	100.0/29.7	2.8/100.0

[a] Not detectable.

with determination of the intensity of the ion at m/z = 87. Of the remaining two ion pairs the 98/100 pair was selected for analysis because it represented an ion pair containing a very clearly defined structural component of **1** (Table I).

Analysis by GC/SIM/MS allowed calculation of the proportions of unlabeled **1** and singly and doubly labeled **1** as well as the location of the label at position 6 or 8 in the singly labeled species. Thus, the ratio of M^+, $(M + 2)^+$, and $(M + 4)^+$, after correction for the natural abundance of isotopes, provided the proportion of unlabeled **1** and singly and doubly labeled **1**. The proportions of **1** unlabeled at position 6 was deduced from the intensity of the fragment ion at 98 (which is due to unlabeled **1** and [6-^{18}O]-**1**) compared to the intensity of the ion at 100 (which is due to doubly labeled **1** and [8-^{18}O]-**1**). These values were corrected for the native relative intensities of the fragment ions presented in Table II.

Mechanism of the Cyclization of (*Z*)-6-Nonen-2-one in Vivo. Of the several biosynthetic pathways outlined in Figure 1 for the conversion of **2** to **1**, one general route can be ruled out on the basis of stereochemical precedent. All epoxide hydrolases studied to date catalyze the S_N2 cleavage of one oxirane carbon–oxygen bond.[11] Thus, if **2** is converted to keto epoxide **3**, and the latter is cleaved by a hydrolase, only threo diol products are expected.

(7) Franke, W. *Mitt. Dtsch. Ges. Allg. Angew. Ent.* **1981**, *2*, 248–251.
(8) Vanderwel, D.; Gries, G.; Singh, M. S.; Borden, J. H.; Oehlschlager, A. C. *J. Chem. Ecol.*, in press.
(9) Gore, W. E.; Pearce, G. T.; Silverstein, R. M. *J. Org. Chem.* **1976**, *41*, 607–610. Pearce, G. T.; Gore, W. E.; Silverstein, R. M. *J. Magn. Reson.* **1977**, *27*, 497–507. Silverstein, R. M.; Brownlee, R. G.; Bellas, T. E.; Wood, D. L.; Browne, L. E. *Science* **1968**, *159*, 889–891.
(10) Vederas, J. C. *Nat. Prod. Rep.* **1987**, 277–337.
(11) Armstrong, R. N. *CRC Crit. Rev. Biochem.* **1987**, *22*, 39–88. Meijer, J.; DePierre, J. W. *Chem.-Biol. Interact.* **1988**, *64*, 207–249.

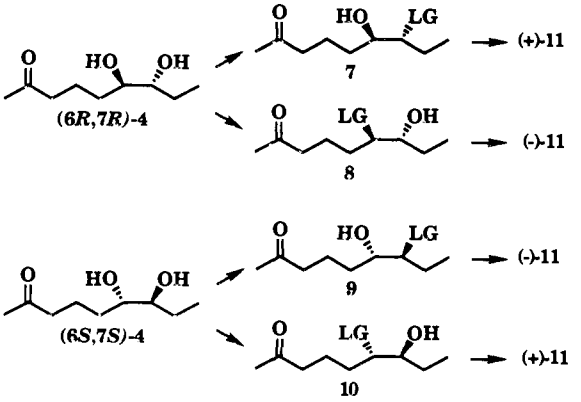


Figure 3. Derivatives (**7**–**10**) of the two enantiomers of *threo*-2-oxo-6,7-nonanediol (**4**), and the expected brevicomin product of cyclization.

There are two enantiomers of *threo*-2-oxo-6,7-nonanediol, (6*R*,7*R*)-**4** and (6*S*,7*S*)-**4**, Figure 3, and each of these could be derivatized at one hydroxyl (**7**–**10**, Figure 3). The usual ketalization process leading to brevicomins would be initiated by S_N2 displacement of the derivatized hydroxyl by the carbonyl. As shown in Figure 4 for the threo diol **7**, the products of such cyclizations are the enantiomers of *endo*-brevicomin (**11**, Figure 3). *endo*-Brevicomin is also produced if the sequence of events involves initial attack of the carbonyl by the underivatized hydroxyl of a threo diol followed by displacement of the derivatized hydroxyl by the intermediate alkoxide. Since **2** is converted to (+)-**1** in vivo,[8] pathways involving threo diols wherein one hydroxyl is displaced by the carbonyl oxygen can be ruled out.

It is possible to differentiate between pathways involving keto epoxide and keto diol intermediates (Figure 1) by determining the number of oxygen atoms in **1** that are supplied by molecular oxygen. This can be done by allowing the biosynthesis of **1** to proceed in the presence of [^{18}O]oxygen gas. In the conversion of an unknown precursor **12** to the keto epoxide **3**, both the carbonyl and the epoxide oxygen of **3** should be supplied by molecular oxygen (Figure 5). Thus, in the presence of $^{18}O_2$, newly biosynthesized **3** should be doubly labeled with oxygen-18. In the event that no exchange occurs between the carbonyl oxygen of **3** and water, cyclization via the keto epoxide should lead to [$^{18}O_2$]-**1**. If, however, cyclization does not proceed directly from [$^{18}O_2$]-**3**, but rather proceeds through a keto diol intermediate, then one of the oxygens of **1** will be derived from molecular oxygen and the other will be derived from the water mediating diol formation. The normal process for conversion of a keto diol to a ketal involves loss of the carbonyl oxygen (Figure 6), so only diol-derived oxygens reside in the final product (i.e., the carbonyl oxygen is lost, Figure 5). Thus [$^{18}O_1$]-**1** will be produced.

Incorporation of $^{18}O_2$ in Vivo. We have previously determined[8] that male MPBs produce (+)-**1** in vivo and that their exposure to vapors of **2** increases production of this pheromone. Thus, with this sex it is possible to study the incorporation of ^{18}O into (+)-**1** from $^{18}O_2$ in the presence and absence of added **2**. Female MPBs do not normally produce **1** but can be induced to produce it by exposure to **2**. In this instance, study of the incorporation of ^{18}O into **1** from $^{18}O_2$ requires simultaneous exposure to **2**.

exo-Brevicomin synthesized by male MPBs in the presence of $^{18}O_2$ was significantly enriched in ^{18}O (Table III). After exposure to $^{18}O_2$ for 24 h, MPBs produced **1** which was ~21% enriched[12] with two atoms of ^{18}O. This increased to ~56% after exposure for 48 h. This result clearly indicates that both oxygens of **1** were derived from molecular oxygen and eliminates biosynthesis of **1** through a keto diol intermediate. Therefore, cyclization must proceed directly from a keto epoxide intermediate.[13]

(12) Enrichment can be calculated according to Campbell: Campbell, I. M. *Bioorg. Chem.* **1974**, *3*, 386–397.

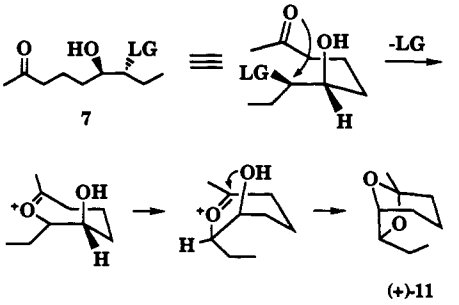


Figure 4. Expected stereochemical outcome of the cyclization of a derivatized form of 2-oxo-6,7-nonanediol, **7**, (a putative biosynthetic derivative of [^{18}O]-**2**) to *endo*-brevicomin. (LG denotes leaving group.)

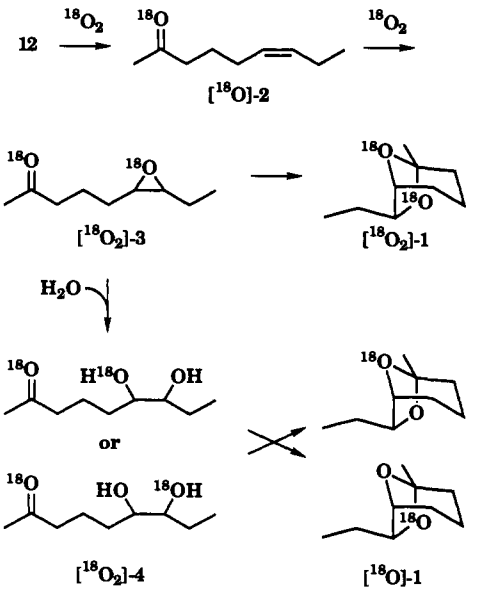


Figure 5. Expected ^{18}O-labeling pattern in **1** formed via 6,7-epoxynonan-2-one (**3**) or 6,7-dihydroxynonan-2-one (**4**) intermediates in the presence of $^{18}O_2$.

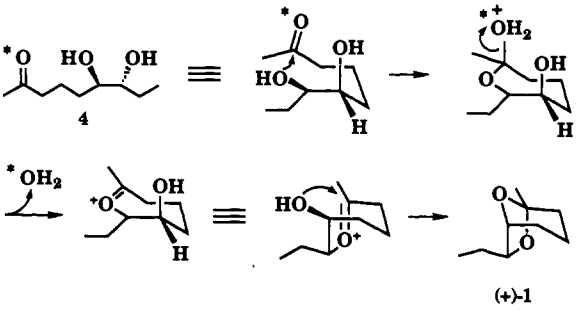


Figure 6. Loss of carbonyl oxygen during cyclization of (6*R*,7*R*)-**4** to **1**.

Table III. Incorporation of ^{18}O into **1**[a] by Male and Female MPBs Exposed to $^{18}O_2$

treatment	sex	no. of samples[b]	relative abundance ($\bar{x}$ ± SE)		
			M^+	$(M+2)^+$	$(M+4)^+$
$^{16}O_2$ (24 h)	male	2	100.0	0.7 ± 0.1	nd
$^{18}O_2$ (24 h)	male	4	100.0	66 ± 12	43 ± 13
$^{18}O_2$ (48 h)	male	1	100.0	262.3	463.1
$^{18}O_2$ + **2** (24 h)	male	7	100.0	307 ± 15	27 ± 2
$^{18}O_2$ + **2** (24 h)	female	5	100.0	278 ± 70	2 ± 1

[a] The (*Z*)-6-nonen-2-one and (*Z*)-6-nonen-2-ol isolated from the beetles were not enriched in ^{18}O. [b] Each sample was prepared from 1–10 beetles.

Relatively little of the (+)-*exo*-brevicomin synthesized by male and female MPBs simultaneously exposed to both $^{18}O_2$ and **2** was

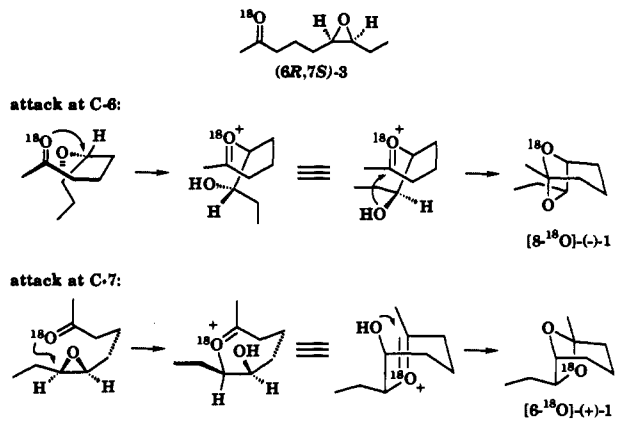


Figure 7. Stereochemical outcome of the cyclization of (6*R*,7*S*)-**3** to **1** through attack of C-6 or C-7 by the ^{18}O carbonyl oxygen.

doubly labeled with ^{18}O (Table III).[14] This is consistent with cyclization proceeding through a keto epoxide, **3**, with incorporation of ^{16}O of the carbonyl into **1**.

Stereochemistry of the Cyclization of the Keto Epoxide Intermediate. The preceding clearly indicates that the conversion of **2** to **1** proceeds via a keto epoxide. It was required to deduce the absolute stereochemistry of the process without administering the enantiomers of the intermediate keto epoxides, since they are sufficiently reactive that their spontaneous cyclization (facilitated by moderate heat or by adventitious acid present in the insects or during isolation) would be virtually impossible to prevent.

It is possible to derive four enantiomers of 6,7-epoxynonan-2-one from **2**. The two threo enantiomers can be eliminated from consideration, because all known laboratory and enzymatic epoxidations of alkenes proceed with retention of the geometry about the olefinic bond. The *Z* geometry of the olefinic linkage in **2** will thus give rise only to the enantiomers of the erythro keto epoxide.

The relationship between the chirality of **1** produced by the MPBs and the chirality of the erythro keto epoxide precursor is determined by which carbon of the epoxide (C-6 or C-7) is attacked by the carbonyl oxygen during cyclization.[15] This is shown for (6*R*,7*S*)-**3** in Figure 7. We have previously shown that MPB produces (+)-**1** under natural conditions and when exposed to deuterated **2**;[8] therefore routes to (−)-**1** can be eliminated from consideration. The two possible routes to (+)-**1** from the enantiomers of **3** involve the cyclization of (6*S*,7*R*)-**3** by initial attack of the carbonyl oxygen at C-6 and cyclization of (6*R*,7*S*)-**3** by initial attack at C-7.

It is possible to distinguish between these possibilities by determination of the position of the ^{18}O label in **1** which originally resided in the carbonyl oxygen of **2**. If the cyclization occurs through (6*R*,7*S*)-**3** with initial attack by the carbonyl carbon at C-7, then the ^{18}O label originally in the carbonyl oxygen of **3** will be in position 6 of (+)-**1** (Figure 7). Similarly, if the cyclization occurs through (6*S*,7*R*)-**3** with initial attack by the carbonyl oxygen at C-6, then the ^{18}O label of the carbonyl oxygen of **3** will be in position 8 of (+)-**1**. The position of the ^{18}O and hence the absolute stereochemistry of the keto epoxide precursor can be discerned from the MS fragmentation pattern as discussed above.

When male MPBs were exposed to vapors of [^{18}O]-**2**, it was found by GC/SIM/MS that the (+)-**1** produced was not enriched in ^{18}O. Since carbonyl oxygens are known to undergo acid-catalyzed exchange with water, we examined recovered **2** and its MPB-produced alcohol reduction product, (*Z*)-6-nonen-2-ol, **13**, for ^{18}O content. The **2** isolated from the insects after the 24-h exposure period no longer had detectable amounts of ^{18}O, while the corresponding alcohol **13** was only ~38% enriched in ^{18}O. Thus, it is very likely that the ^{18}O in **2** was exchanged with unlabeled water in the beetles prior to cyclization to **1**.

To generate ^{18}O-labeled **2** for in vivo cyclization to (+)-**1**, it seemed appropriate to reverse the above process by exposing the beetles to $^{18}OH_2$ and allowing the ^{18}O to exchange into unlabeled **2**. When MPBs were exposed to both $^{18}OH_2$ and unlabeled **2**, the precursor and its alcohol reduction product (**13**) isolated from the beetles were enriched in ^{18}O by ~50% and 38%, respectively. Thus, exchange of ^{18}O into the carbonyl oxygen of **2** occurred.

The (+)-**1** produced by each sex of MPBs simultaneously exposed to both $^{18}OH_2$ and unlabeled **2** was significantly enriched in one ^{18}O/molecule (Table IV). *exo*-Brevicomin produced by male MPBs in the presence of $^{18}OH_2$ but not exposed to unlabeled **2** was also enriched for one ^{18}O/molecule (Table IV). This is presumably due to the exchange of ^{18}O into endogenous **2**.

Analysis by GC/SIM/MS of the (+)-**1** produced by MPBs exposed to $^{18}OH_2$ with and without added **2** revealed ^{18}O in both

Table IV. Incorporation of ^{18}O into **1** by Male and Female MPBs Exposed to $^{18}OH_2$ for 24 h: Relative Abundance of Peaks of Interest (GC/SIM/MS)

treatment	sex	no. of samples[a]	relative abundance ($\bar{x}$ ± SE) M^+	$(M+2)^+$	$(M+4)^+$
$^{16}OH_2$	male	2	100.0	0.7 ± 0.1	nd
$^{18}OH_2$	male	2	100.0	17 ± 2	nd
$^{18}OH_2$ + **2**	male	3	100.0	65 ± 35	nd
$^{18}OH_2$ + **2**	female	1	100.0	25	nd

[a] Each sample was usually prepared from five beetles.

Table V. Incorporation of ^{18}O from $^{18}O_2$ into **1** by MPB: Relative Ratios of [6-^{18}O]-**1** to [8-^{18}O]-**1** Produced

treatment	sex	no. of samples	ratios ($\bar{x}$ ± SE) [6-^{18}O]-**1**	[8-^{18}O]-**1**
$^{18}OH_2$	male	1[a]	1.0	1.1
$^{18}OH_2$ + **2**	male	2[b]	1.0	4.6 ± 0.5
$^{18}OH_2$ + **2**	female	1[c]	1.8	1.0

[a] Sample prepared from six beetles. [b] Each sample prepared from five beetles. [c] Sample prepared from five beetles.

Table VI. Incorporation of ^{18}O from $^{18}O_2$ into **1** by Male and Female MPBs Exposed (or Not Exposed) to **2** for 24 h: Relative Ratios of [6-^{18}O]-**1** to [8-^{18}O]-**1** Produced

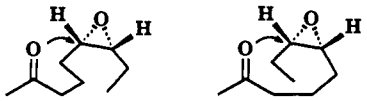
expt no.	treatment	sex	no. of samples	ratios ($\bar{x}$ ± SE) [6-^{18}O]-**1**	[8-^{18}O]-**1**
1	$^{18}O_2$	male	4[a]	2.4 ± 0.7	1.0
	$^{18}O_2$ + **2**	male	4[a]	2.5 ± 0.1	1.0
	$^{18}O_2$ + **2**	female	4[a]	1.0	5.4 ± 2.1
2	$^{18}O_2$	male	2[b]	1.6 ± 0.0	1.0
	$^{18}O_2$ + **2**	male	2[c]	1.5 ± 0.2	1.0
	$^{18}O_2$ + **2**	female	2[a]	1.0	2.0 ± 0.3
3	$^{18}O_2$	male	2[d]	3.6 ± 1.1	1.0
	$^{18}O_2$ + **2**	male	5[d]	2.6 ± 0.3	1.0
	$^{18}O_2$ + **2**	female	3[d]	1.0	3.5 ± 0.6

[a] Each sample prepared from 10 beetles. [b] Each sample prepared from 20 beetles. [c] Each sample prepared from five beetles. [d] Each sample prepared from one beetle.

(13) The basis of this interpretation is the assumption that [$^{18}O_2$]-**1** was produced in the presence of $^{18}O_2$, but not $^{18}OH_2$. We do not believe that significant quantities of $^{18}OH_2$ would form in vivo in the presence of $^{18}O_2$, since the oxygen-18 of oxygen gas will not spontaneously exchange with the oxygen of water. Very small quantities of $^{18}OH_2$ might form in vivo through exchange of the oxygens of water with those of ^{18}O-labeled ketones (formed in vivo through oxidase activity in the presence of $^{18}O_2$). However, one would not expect this process to contribute significantly to the production of ^{18}O-labeled **1**. Data presented in this paper support this assumption. Firstly, when the beetles were directly exposed to relatively large quantities of ^{18}O-labeled ketone **2**, incorporation of oxygen-18 into **1** was not detectable. Secondly, even when the beetles were directly exposed to relatively large quantities of $^{18}OH_2$, the **1** produced by control male beetles was only ~14% enriched in oxygen-18 (Table IV). In contrast, the **1** produced by control (untreated) male beetles after 48 h was ~56% enriched for two atoms of oxygen-18 (Table III). Thus we believe that $^{18}OH_2$ formed in vivo would make a negligible contribution to the ^{18}O-labeling of **1**.

(14) The $(M+4)^+$ peak of the **1** produced by the females is 0.7 ± 0.5% the abundance of the $(M+2)^+$ peak. This is largely due to the natural abundance of heavy isotopes in molecules of **1** singly enriched for ^{18}O, rather than enrichment for two ^{18}O atoms/molecule of **1**.

(15) Wasserman, H. H.; Wolff, S.; Oku, T. *Tetrahedron Lett.* **1986**, *27*, 4909–4912. Wasserman, H. H.; Oku, T. *Tetrahedron Lett.* **1986**, *27*, 4913–4916.



(6S,7R)-3 **(6R,7S)-3**

Figure 8. Example of possible stereochemistry of the initial steps in the cyclization of (6*S*,7*R*)-**3** and (6*R*,7*S*)-**3** ultimately leading to (+)-**1**.

positions (Table V). Incorporation of label into both positions 6 and 8 of (+)-**1** indicates that the cyclization proceeded through both enantiomers of **3**. Most of the label in the (+)-**1** produced by male MPBs exposed to $^{18}OH_2$ with or without **2** was in position 8, indicating that cyclization of (6*S*,7*R*)-**3** was favored over cyclization of (6*R*,7*S*)-**3**. Surprisingly, most of the (+)-**1** produced by female MPBs when exposed to $^{18}OH_2$ and unlabeled **2** was ^{18}O-labeled at position 6, suggesting that, in this sex, production of (+)-**1** was via cyclization of (6*R*,7*S*)-**3**.

These interpretations were confirmed by studying the incorporation of ^{18}O from $^{18}O_2$ into (+)-**1** in male and female MPBs in the absence and/or presence of **2**. As revealed in Table VI, the ^{18}O label in singly labeled **1** produced by both sexes was once again found in both positions. In the case of males, more label was found in position 6 than in position 8, which is consistent with the preferential formation of (+)-**1** from (6*S*,7*R*)-**3** containing ^{18}O in the epoxide oxygen rather than the carbonyl oxygen. When female MPBs were exposed to $^{18}O_2$ and **2**, the ^{18}O label in singly labeled (+)-**1** was found primarily at position 8 (Table VI). This result is consistent with the preferential cyclization of (6*R*,7*S*)-**3**.

In the case of males, the majority of ^{18}O label was located at position 6 of **1** when the exposure was conducted both in the presence and in the absence of precursor (**2**). This observation provides further evidence[8] that the latter is a true intermediate in the biosynthesis of **1** in MPBs.

All samples discussed above were prepared from several beetles. To determine if individual insects produced (+)-**1** from (6*R*,7*S*)-**3** while others utilized (6*S*,7*R*)-**3** we analyzed (+)-**1** obtained from individual males that had been exposed to $^{18}O_2$ both in the presence and in the absence of **2**. We also analyzed extracts of individual female MPBs that had been exposed to both $^{18}O_2$ and **2** (Table VI). Extracts from both sexes contained (+)-**1** which was singly labeled with ^{18}O in both possible positions. Thus, single insects use both enantiomers of **3** to produce (+)-**1**. The proportions of label in positions 6 and 8 of (+)-**1** were in agreement with the view that (6*S*,7*R*)-**3** was the preferred precursor in males while its enantiomer was the preferred precursor in females.

Concluding Remarks

The above represent prima facie evidence that in MPBs both (6*S*,7*R*)-**3** and (6*R*,7*S*)-**3** are transformed to (+)-**1**. The mechanism of cyclization is required to involve attack of the oxirane ring by the carbonyl oxygen as shown in Figure 7. This situation is analogous to that reported by Prestwich et al.[16] regarding the hydrolysis of disparlure, the epoxide pheromone of the gypsy moth, by enzymes located in the antennae of the male. The hydrolysis of both (+)- and (−)-disparlure led to the same (7*R*,8*R*)-*threo*-diol. As shown in Figure 8, the cyclizations of (6*S*,7*R*)-**3** and (6*R*,7*S*)-**3** could, similarly, be considered to be catalyzed by a single enzyme. The active site of the enzyme might lock the oxygens of the two enantiomers of **3** into the same relative stereochemistry, while the hydrocarbon chains fold into a hydrophobic pocket in the enzyme. Attack of the oxirane ring by the carbonyl oxygens would result in the cyclization of both enantiomers of *erythro*-**3** to (+)-**1**.

Experimental Section

General. Gas–liquid chromatographic analyses were conducted on a Hewlett-Packard 5890A gas chromatograph fitted with a capillary inlet system, flame-ionization detector, and DB-1 column (15 m × 0.25 mm i.d., 0.25-μm film thickness) of fused silica. The oven temperature program was as follows: 50 °C for 1 min, then 4 °C/min to 80 °C for 10 min, followed by an increase at 25 °C/min to a final temperature of 275 °C for 10 min. Injection port and detector temperatures were 260 and 275 °C, respectively. Helium was the carrier gas.

Mass spectra were obtained on a Hewlett-Packard 5985B gas chromatograph–mass spectrometer by electron impact (EI, 70 eV) or by chemical ionization (CI, isobutane). Single ion monitoring (GC/SIM/MS) analyses were performed as previously described.[17] ^1H and ^{13}C NMR spectra were recorded at 400.13 and 100.6 MHz, respectively, in $CDCl_3$ on a Bruker 400 WM spectrometer. Signal positions are given in δ units. Infrared spectra (IR) were recorded neat (NaCl disk) on a Perkin-Elmer Model 599B spectrometer.

[^{18}O]Water ($^{18}OH_2$, 97% ^{18}O) was purchased from Merck Sharp & Dohme/Isotopes. [^{18}O]Oxygen gas ($^{18}O_2$, >97% ^{18}O) was purchased from Isotec Inc. Pherotech, Inc. (Delta, BC, Canada) generously donated (*E*)- and (*Z*)-6-nonen-2-one.

MPBs were reared on lodgepole pine bolts, and their sex was determined as previously described.[8]

Treatment of Insects. A. Exposure to $^{18}O_2$. The void volumes of serum-stoppered Schlenk tubes containing glass beads (to provide a walking surface for the beetles and to minimize fighting) were first determined (~35 mL). Tubes were then evacuated under high vacuum, and $^{18}O_2$ was added by gas-tight syringe to $^1/_5$ atm as calculated from the void volume. Nitrogen was then added to atmospheric pressure. Beetles (usually 12/tube) were quickly introduced into each tube by carefully lifting off the septum. Extra $^{18}O_2/N_2$ (1/5, v/v) was continuously blown into the tube until the septum was replaced. Beetles were left in the $^{18}O_2/N_2$ atmosphere in the dark at room temperature for the periods of time specified in Results and Discussion. As required, **2** was administered by syringe to the tubes at 0.1 μL/3.5 mL of void volume. After the exposure period, whole beetles were placed on dry ice and extracted with ~400 μL of hexane.[8]

B. Exposure to $^{18}OH_2$. To partially dehydrate male MPBs, they were first held on dry Kimwipes in a small, loosely capped jar for 24 h in the dark. After this treatment they were transferred to a vial containing a piece of Kimwipe soaked in ~200 μL of $^{18}OH_2$ and held for 48 h. Beetles to be treated with **2** were not subjected to prior dehydration but were transferred directly from pine bolts to vials containing $^{18}OH_2$, in which they were held for 48 h. After this treatment they were exposed individually to the vapors of 0.1 μL of **2** in 3.5-mL screw-cap vials for 24 h.[18] Insects were then extracted with hexane as described above.

Syntheses of Chemicals: Synthesis of [2-^{18}O]-(*Z*)-6-Nonen-2-one. A. 2-[(*Z*)-4-Hepten-1-yl]-2,5,5-trimethyl-1,3-dioxane (5). A toluene solution (60 mL) containing **2** (10.7 mmol), 2,2-dimethyl-1,3-propanediol (11.3 mmol), and a catalytic quantity of *p*-toluenesulfonic acid (0.011 mmol) was refluxed overnight. Water was removed as it formed by Dean–Stark extraction. The solution was cooled and extracted with a saturated solution of $NaHCO_3$. The organic extract was dried over anhydrous K_2CO_3. Distillation under vacuum yielded a colorless oil (2.29 g, 95% yield), bp 89–92 °C (1.2 mmHg), 96% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 0.89 (3 H, d, $J_{CH2,CH3}$ = 1.2 Hz, ketal methyl), 0.95 (3 H, dt, $J_{8,9}$ = 7.5 Hz, $J_{7,9}$ = 1.2 Hz, 9-H), 1.00 (3 H, s, acetal methyl), 1.35 (3 H, d, *J* = 2.9 Hz, 1-H), 1.42–1.51 (2 H, m, 4-H), 1.65–1.72 (2 H, m, 3-H), 1.99–2.08 (4 H, m, 5-H and 8-H), 3.43 (2 H, dd, J_{gem} = 11 Hz, $J_{CH2,CH3}$ = 1.2 Hz, acetal methylenes), 3.53 (2 H, d, J_{gem} = 11 Hz, acetal methylenes); 5.28–5.40 (2 H, m, 6-H and 7-H). MS (EI) *m/z* (relative intensity): 226 (M^+, 5), 211 (50), 129 (100), 82 (10), 69 (30).

B. [2-^{18}O]-(*Z*)-6-Nonen-2-one ([^{18}O]-2). Gaseous HCl was bubbled through a vigorously stirred mixture of hexane (200 μL, previously dried over Na wire) and $^{18}OH_2$ (200 μL) for ~10 s. Compound **5** (4.0 mmol) was added neat (a small portion of anhydrous hexane was used to complete the transfer) with cooling. The mixture was stirred at room temperature, and the progress of the reaction was monitored by periodic removal and GC analysis of aliquots. The reaction was essentially complete in 4 h. Anhydrous hexane (~0.5 mL) was added, and the aqueous and organic phases were separated. The organic phase was passed through a short column of anhydrous K_2CO_3. The aqueous phase was washed with anhydrous hexane (6 × 50 μL); the washings were passed through the column. The combined hexane extracts were distilled from K_2CO_3 using a 2.5-cm Vigreux column. The hexane was first removed at atmospheric pressure; then a vacuum was applied to distill the [^{18}O]-**2** (101–102 °C, 34 mmHg), which was obtained as an oil (0.32 g, 56% yield), 98% pure by GC. ^1H NMR ($CDCl_3$): δ 1.05 (3 H, t, 9-H), 1.64–1.73 (2 H, m, 4-H), 1.96–2.07 (4 H, m, 5-H and 8-H), 2.12 (3 H, s, 1-H), 2.43 (2 H, t, 3-H), 5.23–5.31 (1 H, m, 7-H), 5.35–5.44 (1 H, m, 6-H). MS (EI) *m/z* (relative abundance): 142 (M^+, 18), 127 (12),

(16) Prestwich, G. D.; Graham, S. McG.; König, W. A. *J. Chem. Soc., Chem. Commun.* **1989**, 575–577.

(17) Vanderwel, D.; Johnston, B.; Oehlschlager, A. C. Submitted to *Insect Biochem.*

(18) Gries, G.; Bowers, W. W.; Gries, R.; Noble, M.; Borden, J. H. *J. Insect Physiol.* **1990**, *36*, 819–824.

113 (20), 82 (86), 67 (100), 60 (12), 55 (14), 45 (50). The product was 94% ^{18}O by GC/SIM/MS analysis.

Synthesis of [8-^{18}O]-*exo*-Brevicomin. A. 2-[(*Z*)-4,5-Epoxyhept-1-yl]-2,5,5-trimethyl-1,3-dioxane (6). A solution of **5** (2.2 mmol) in CH_2Cl_2 (20 mL) was added to a vigorously stirred solution of $NaHCO_3$ (3.6 mmol) in water (7 mL). *m*-Chloroperbenzoic acid (MCPBA) (2.7 mmol) was added, and the biphasic system was stirred vigorously at room temperature overnight. Workup yielded a colorless oil (0.55 g, quantitative yield), 87% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 0.88 (3 H, s, acetal methyl), 1.02 (3 H, s, acetal methyl), 1.04 (3 H, t, $J_{8,9}$ = 8 Hz, 9-H), 1.37 (3 H, s, 1-H), 1.47-1.78 (8 H, M, 3-H, 4-H, 5-H, 8-H), 2.84-2.90 (1 H, m, 7-H), 2.90-2.96 (1 H, m, 6-H), 3.42 (2 H, d, J_{gem} = 12 Hz, acetal methylenes), 3.56 (2 H, d, J_{gem} = 12 Hz, acetal methylenes). MS (EI) *m/z* (relative intensity): 227 (75), 129 (100), 69 (30).

B. [8-^{18}O]-*exo*-Brevicomin ([8-^{18}O]-1). Compound **6** prepared above (1.9 mmol), dissolved in anhydrous diethyl ether (~200 μL), was added to $^{18}OH_2$ (0.5 mL) through which gaseous HCl had been bubbled for ~10 s. The reaction mixture was stirred vigorously at room temperature, during which time aliquots were periodically removed and analyzed by GC. Gaseous HCl was periodically bubbled through the reaction mixture as needed to maintain a satisfactory reaction rate. After 12 h, the reaction was essentially complete. The two phases were separated, and the aqueous phase was extracted with 3 × 0.1 mL of anhydrous diethyl ether. The combined ether extracts were concentrated by blowing a gentle stream of argon over them. Bulb-to-bulb distillation under vacuum yielded crude ^{18}O-**1** (0.38 g) which was 70% pure by GC analysis. Purification by distillation under vacuum (69-72 °C, 20 mmHg) gave [^{18}O]-**1** as a colorless oil (0.25 g, 76% yield), which was 95% pure by GC analysis. ^1H NMR ($CDCl_3$): δ 0.91 (3 H, distorted t, $J_{10,11}$ = 7.5 Hz, 11-H), 1.41 (3 H, s, 9-H), 1.43-1.95 (8 H, m, 2-H, 3-H, 4-H, and 10-H), 3.93 (1 H, t, $J_{7,10}$ = 6.5 Hz, 7-H), 4.13 (1 H, br s, 1-H). ^{13}C NMR ($CDCl_3$): δ 9.61 (11-C), 17.15 (3-C), 25.00 (9-C), 28.00 and 28.58 (2-C and 10-C), 35.03 (4-C), 78.63 (1-C), 81.49 (7-C), 108.19 (5-C). MS (EI) *m/z* (relative intensity): 158 (M^+, 14), 129 (18), 116 (17), 114 (80), 101 (11), 100 (26), 88 (21), 87 (17), 85 (100), 81 (16), 72 (12), 68 (22), 67 (16), 45 (26), 43 (18).

The position of the ^{18}O label in the product was deduced from the ^{18}O-isotope-induced shifts of the ^{13}C NMR signals, after resolution enhancement of the ^{13}C NMR spectrum (line broadening, -0.5 Hz; Gaussian broadening, 0.3; 64K data set). The results are presented in Results and Discussion.

Registry No. (+)-**1**, 20290-99-7; [8-^{18}O]-**1**, 141171-85-9; (*Z*)-**2**, 34019-86-8; [^{18}O]-**2**, 141171-83-7; (6*S*,7*R*)-**3**, 141269-69-4; (6*R*,7*S*)-**3**, 141269-70-7; (*Z*)-**5**, 141171-84-8; (*S*,*R*)-**6**, 141269-71-8.

Cationic Metallovesicles: Catalysis of the Cleavage of *p*-Nitrophenyl Picolinate and Control of Copper(II) Permeation

Paolo Scrimin,* Paolo Tecilla, and Umberto Tonellato*

Contribution from the Department of Organic Chemistry and Centro CNR Meccanismi di Reazioni Organiche, University of Padova, Via Marzolo, 1, 35131 Padova, Italy.
Received September 3, 1991

Abstract: Ligand surfactants **1-3** have been synthesized. They feature a 2-(hydroxymethyl)pyridine as the ligand subunit and different lipophilic backbones which allow the formation, in aqueous solution, of different aggregates: vesicles in the case of **1** and **2**, micelles in the case of **3**. In the presence of Cu^{2+} ions, these aggregates are catalysts of the hydrolytic cleavage of activated esters of amino acids. Those made of surfactants **1** and **2** constitute one of the first examples of hydrolytic metallovesicles. The kinetic investigation was focused on the hydrolysis of the *p*-nitrophenyl ester of picolinic acid (PNPP): in line with previous results, the kinetic effects were attributed to the pseudo-intramolecular transacylation of a metal-ion-coordinated hydroxy group of the ligand subunit via the formation of a ternary complex comprising surfactant, Cu^{2+}, and PNPP. The observed accelerations were shown to be strongly dependent on the nature of the aggregate and, for vesicles, on the structure of the lipophilic backbone. This modulation of the reactivity has been attributed to (i) the different tendencies of the ligand amphiphiles in the aggregates to form nonproductive ternary complexes involving two ligand molecules and one Cu^{2+} ion and (ii) the rate of flip-flop of the surfactants which is suggested to be responsible for the transport of the Cu^{2+} ions across the vesicular membrane.

Synthetic liposomes[1] are the focus of the research interest of an increasing number of laboratories, as they may give information on critical phenomena pertaining to biological membranes.[2] The unique characteristics of a vesicular membrane, remarkably different from those of other assemblies, allow, for instance, the study of neutral and ionic molecular permeation,[3a] mobility of the surfactants in the bilayer,[3b] and catalytic efficiency[4] (in functional[5] aggregates). Furthermore, surface differentiation is possible, and indeed, several surface-differentiating reactions have been reported.[6] For instance, Moss and his associates have achieved chemical differentiation between the internal and external surfaces of a vesicle[7] through selective esterolysis. These authors have also shown how the intrabilayer movement (flip-flop) of the surfactant is dramatically dependent on the structure of the lipophilic backbone.[8]

The exciting outcome of new results in this area prompted us to broaden our interest on metalloaggregates[9] as mimics of hy-

(1) Fendler, J. H. *Membrane Mimetic Chemistry*; Wiley: New York, 1982; Chapter 6.
(2) Jain, M. *Introduction to Biological Membranes*, 2nd ed.; Wiley: New York, 1988.
(3) (a) Reference 2, Chapter 9. (b) Ibid., Chapter 4.
(4) Review of functional vesicles: Fuhrhop, J.-H.; Mathieu, J. *Angew. Chem., Int. Ed. Engl.* **1984**, *23*, 100.
(5) (a) Moss, R. A.; Scrimin, P.; Bhattacharya, S.; Swarup, S. *J. Am. Chem. Soc.* **1987**, *109*, 6209. (b) Moss, R. A.; Ganguli, S. *Tetrahedron Lett.* **1989**, *30*, 2071. (c) Kunitake, T.; Ihara, H.; Okahata, Y. *J. Am. Chem. Soc.* **1983**, *105*, 6070. (d) Murakami, Y.; Nakano, A.; Yoshimatsu, A.; Fukuya, K. *J. Am. Chem. Soc.* **1981**, *103*, 728.
(6) (a) Fuhrhop, J.-H.; Bartsch, H.; Fritsch, D. *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 804. (b) Tundo, P.; Kurihara, K.; Kippenberger, J.; Politi, M.; Fendler, J. H. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 81. (c) Mizutani, T.; Whitten, D. G. *J. Am. Chem. Soc.* **1985**, *107*, 3621. (d) Kunitake, T.; Okahata, Y.; Yasunami, S. *J. Am. Chem. Soc.* **1982**, *104*, 5547.
(7) (a) Moss, R. A.; Bhattacharya, S.; Scrimin, P.; Swarup, S. *J. Am. Chem. Soc.* **1987**, *109*, 5740. (b) Moss, R. A.; Swarup, S. *J. Am. Chem. Soc.* **1986**, *108*, 5341.
(8) (a) Moss, R. A.; Bhattacharya, S.; Chatterjee, S. *J. Am. Chem. Soc.* **1989**, *111*, 3680. (b) Moss, R. A.; Ganguli, S.; Okumura, Y.; Fujita, T. *J. Am. Chem. Soc.* **1990**, *112*, 6391.

0002-7863/92/1514-5086$03.00/0