A Combinatorial Library of Macrocyclic Polyamines Produced by a Ladybird Beetle

Frank C. Schroeder,*,† Jay J. Farmer,† Scott R. Smedley,‡ Athula B. Attygalle,† Thomas Eisner,§ and Jerrold Meinwald†

Contribution from Baker Laboratory, Department of Chemistry and Chemical Biology, Cornell University, Ithaca, New York 14853, Department of Biology, Trinity College, Hartford, Connecticut 06106, and Department of Neurobiology and Behavior, Seeley G. Mudd Hall, Cornell University, Ithaca, New York 14853

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Abstract: The pupal defensive secretion of the coccinellid beetle *Epilachna borealis* is composed principally of a combinatorial library of several hundred macrocyclic polyamines. This new family of natural products is characterized by extremely large-ring monocyclic polylactones, which are derived from the oligomerization of three homologous (*ω*-1)-(2-hydroxyethylamino)alkanoic acids. The characterization and quantification of these novel macrocyclic alkaloids as well as of related open-chain oligomers are described. Via HPLC-MS analyses of suitable derivatives, macrocycles with ring sizes from 24 to over 150 members were characterized. Whereas the three building blocks appear to be randomly incorporated into these oligomers, comparison with other ladybird beetle species suggests that the spectrum of ring sizes produced is carefully regulated.

Introduction

Alkaloids play a major role in the chemical ecology of arthropods. This is especially well documented for several species of ladybird beetles (Coccinellidae)[1] and ants (Formicidae).[2] In most of the alkaloids identified from these arthropods, an unbranched carbon chain is attached at one or more sites to a nitrogen atom, forming linear and mono-, di-, and tricyclic structures. Figure 1 shows two typical examples, propyleine (**1**), present in the hemolymph of several ladybird beetle species,[1] and myrmicarin 217 (**2**), identified from the poison gland secretion of *Myrmicaria* ants.[3]

A number of recent studies have revealed an additional motif in arthropod alkaloid chemistry, the formation of polybasic, oligomeric alkaloids. Analyses of the hemolymph of *Chilocorus*, *Exochomus*, and *Psylloborus* ladybird beetles led to the identification of a new series of "dimeric" alkaloids, for example exochomine (**3**) and psylloborine (**4**), the carbon skeletons of which result from joining two unbranched carbon chains by one or more new carbon−carbon bonds.[4] Structurally related dimeric and trimeric alkaloids such as myrmicarin 663 (**5**) have been identified from the poison gland secretion of *Myrmicaria* ants.[5] In both coccinellid beetles and myrmicine ants, the oligomerization of monobasic bi- and tricyclic alkaloids appears to serve as a simple and effective strategy for the biosynthesis of larger structures. Here we present the details of our study of the pupal defensive secretion of the squash beetle, *Epilachna borealis*, where a related strategy has been exploited to an unprecedented extent.[6] Pupae of this ladybird beetle produce a combinatorial library of macrocyclic alkaloids containing several hundred structures, derived from the oligomerization of only three simple fatty-acid derivatives.

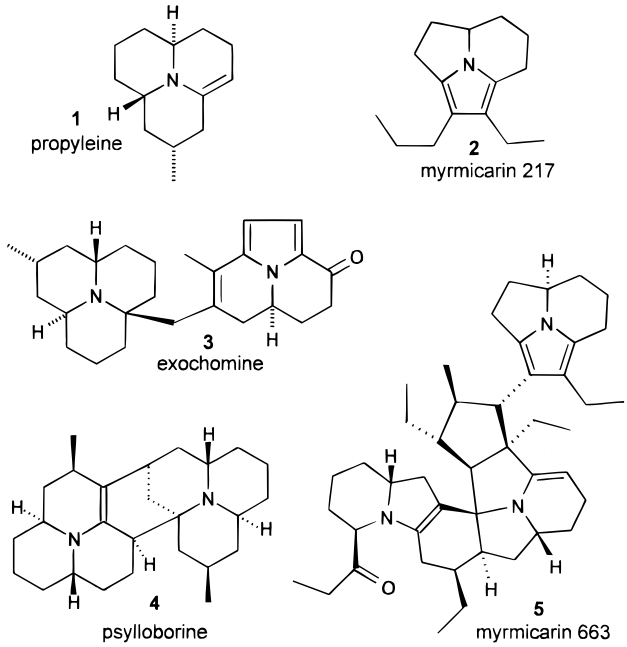

Figure 1. Typical acetogenin alkaloids from ladybird beetles (**1**) and *Myrmicaria* ants (**2**), along with their oligomeric derivatives **3**−**5**.

† Department of Chemistry and Chemical Biology, Cornell University.
‡ Trinity College.
§ Department of Neurobiology and Behavior, Cornell University.

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Table 1. Major Homologous Series of PAML's in the *E. Borealis* Pupal Secretion, Detected by HPLC-MS[a]

	dimers	trimers	tetramers	pentamers	hexamers	heptamers	all higher oligomers
MN	454	681	908	1135	1362	1589	
	440	667	894	1121	1348	1575	
	426	653	880	1107	1334	1561	
		639	866	1093	1320	1547*	
		625	852	1079	1306	1533*	
		611	838	1065	1292*	1519*	
		597	824	1051*	1278*		
			810*	1037*			
			796*				
	<1	42	34	14	6	2	2

[a] MN = nominal molecular mass; an asterisk indicates PAML's detected only via their peracetylated derivatives. [b] The sum is the estimated relative amounts of each series of oligomers, based on the relative abundance of the pseudomolecular ions obtained by HPLC-MS for the peracetylated derivatives.

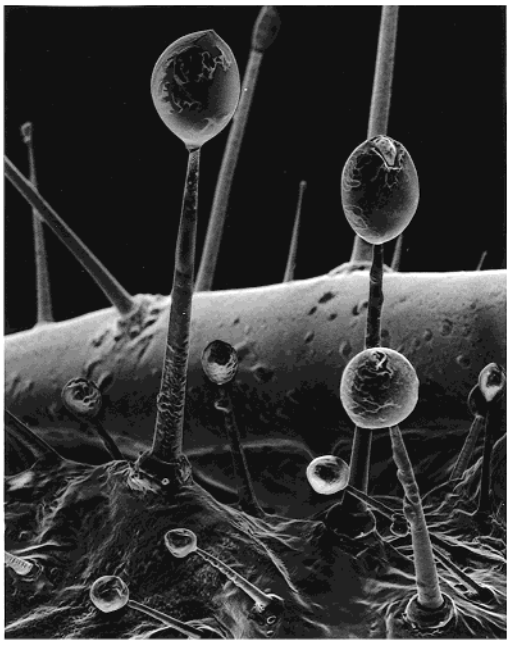

Figure 2. Scanning electron micrograph of the surface of *E. borealis* pupa, showing glandular hairs amid nonglandular bristles (214 ×).

Polyazamacrolides

Epilachna pupae bear a dense coating of glandular hairs that secrete oily droplets deterrent to insects (Figure 2).[7,8] Gas chromatographic analyses of the pupal secretion of the squash beetle, *E. borealis*, resulted in the characterization of vitamin E acetate and other tocopherol derivatives.[8,9] Surprisingly, direct NMR-spectroscopic analyses revealed that the tocopheryl acetates account for only a relatively small percentage of the beetles' total secretion (~20%), while the major components represented a group of previously undetected compounds.[6] These components were shown to be esters and amides derived from the carboxyl- and the 2-hydroxyethylamino moieties of the three (ω-1)-(2-hydroxyethylamino)alkanoic acids, **6**−**8**, which occur in the ratio 2.5:8.5:89 (Figure 3).[6]

From the complexity of the ^{1}H NMR spectra of the secretion, it was apparent that the three homologous (ω-1)-(2-hydroxyethylamino)alkanoic acids **6**−**8** must represent the "building blocks" of a large number of structurally similar cyclic esters and amides.[6] Thus, the new *E. borealis* secretion components appeared to consist of higher molecular weight oligomers, each combining several (ω-1)-(2-hydroxyethylamino)alkanoic acid units. This hypothesis was corroborated by HPLC-MS analyses using positive-ion electrospray ionization, which revealed the secretion to contain a highly convoluted mixture of macrocyclic polyamines (Figure 4).

From the HPLC-MS analyses we discerned three distinct levels of complexity. First, the three building blocks are combined into oligomers of a wide range of molecular weights. The major components are cyclic trimers, tetramers, and pentamers of **6**−**8**, which account for 90% of the total oligomeric mixture. Smaller quantities of dimers, hexamers, and heptamers constitute another 8% (Table 1). Second, each of these groups of oligomers is represented by a series of several homologues. For example, from the three building blocks **6**−**8** one can derive a series of nine sets of homologous cyclic tetramers with nominal molecular masses between 796 and 908 amu. Since compounds with nominal molecular masses corresponding to all nine homologues of this tetrameric series can be detected by HPLC-MS (Table 1), we conclude that the oligomerization process is nonselective with regard to which of the three building blocks are incorporated. Ion chromatograms representing the most abundant five homologous tetramers are shown in Figure 4C. A third level of complexity is revealed by the fact that each of these chromatograms contains several peaks, indicating that in this series of tetrameric homologues each molecular mass value is represented by a group of isomers. Similarly, the members of the other series of oligomers detected in the HPLC-MS analyses also occur in the form of several isomers, of which the first-eluting always appears as the most abundant one. Using repeated preparative HPLC fractionation, the most abundant members of the trimeric, tetrameric, and pentameric series were isolated (Figure 4). One- and two-dimensional ^{1}H NMR spectroscopic analyses showed these components to be the symmetric macrocyclic lactones **10**, **11**, and **12** derived from three, four, or five units of 10-(2-hydroxyethylamino)undecanoic acid (**8**) (Figure 3).[6] Thus, the earliest-eluting, most abundant isomers of each oligomer appear to be the macrocyclic lactones **9**−**14** ("polyazamacrolides" [PAML's]) generated by a nonselective oligomerization of the three building blocks, **6**−**8**.

These structural assignments were confirmed by total syntheses of the polyazamacrolides derived from three, four, five, six, seven, and eleven units of 10-(2-hydroxyethylamino)-undecanoic acid (**8**).[10] The absolute configuration of the

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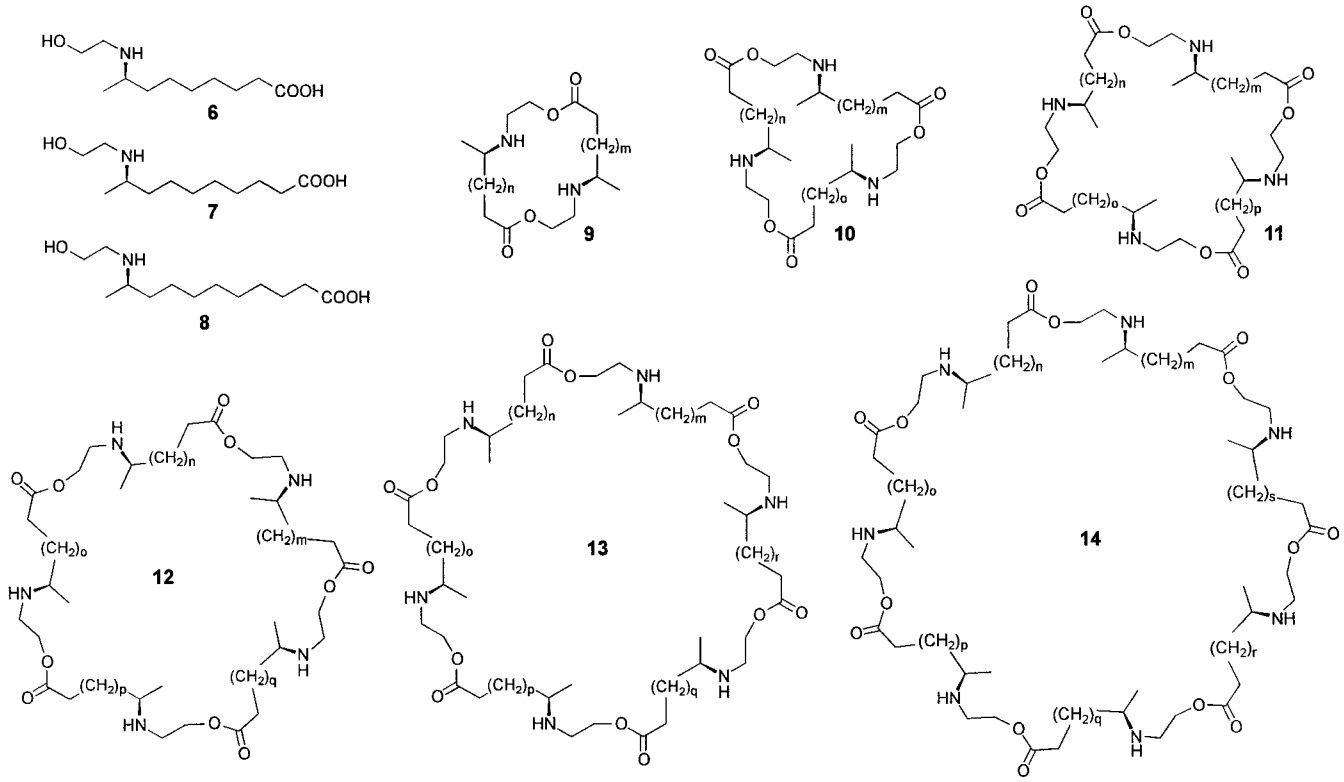

Figure 3. (ω-1)-(2-Hydroxyethylamino)alkanoic acids **6**, **7**, and **8**, the building blocks of the polyazamacrolides (PAML's) **9**−**14** in *E. borealis*. In these formulas, each of the variables *m* to *s* can have the values 5, 6, or 7.

asymmetric center in the three (ω-1)-(2-hydroxyethylamino)-alkanoic acids (**6**−**8**) was determined to be *R* with more than 99% enantiomeric excess (ee).[11]

As shown in Figure 4, HPLC-MS analyses of the pupal secretion indicated the presence of several later-eluting isomers of each of the PAML's, **9**−**14**. These compounds were identified as macrocycles with one or more amide linkages and a correspondingly reduced number of ester linkages.[6] The presence of these macrocyclic lactams in the native secretion apparently results from spontaneous rearrangement of the initially produced completely lactonic PAML's **9**−**14**.[6,12] As illustrated in Figure 5 for the tetramer PAML **908**, this intramolecular rearrangement greatly increases the structural diversity of the library of macrocycles.

Higher Oligomers

One of the more unusual features of the mixture of PAML's in the *E. borealis* pupal secretion is the large range of ring sizes produced. It seemed interesting to analyze the secretion for the presence of oligomers incorporating even more than seven of the hydroxyethylamino acid moieties, and furthermore, to investigate whether there is an "upper limit" to the ring sizes produced by this beetle. As already described for the trimers (**10**), tetramers (**11**), and pentamers (**12**), positive-ion electrospray ionization of the PAML's leads to series of multiply charged pseudomolecular ions, corresponding to the number of basic nitrogens in each component (Figure 6). This tendency to produce families of pseudomolecular ions makes the detection of higher oligomers increasingly difficult, because the mass spectroscopic response is distributed over an increasingly wide *m*/*z* range, while the concentration of higher oligomers in the secretion diminishes rapidly. As would be expected, the intensity of the singly and doubly charged pseudomolecular ions relative to the intensity of more highly charged ions decreases as the number of incorporated building blocks increases, further exacerbating the problem. However, for per-*N*-acetylated derivatives of the PAML's and their lactam isomers, the mass spectrometric conditions can be optimized so that mostly singly or doubly, or in case of oligomers derived from more than eleven building blocks also triply, charged pseudomolecular ions are formed. Consequently, following conversion to their peracetylated derivatives, trace amounts of cyclic oligomers consisting of up to fifteen and more hydroxy amino acid units can be detected, corresponding to ring sizes of above 200 members. Ion chromatograms corresponding to the doubly charged bis-sodium adducts $(M + 2Na)^{2+}$ of series of four homologous octamers, nonamers, and undecamers as well as chromatograms corresponding to the triply charged species $(M + 3Na)^{3+}$ of 13-mer PAML **2951** and 14-mer PAML **3178** are shown in Figure 7. It appears, nevertheless, that while there is no clear upper limit for the oligomer sizes produced, oligomers derived from more than eleven building blocks occur only at very low concentrations.

Quantitative Analyses

Before discussing quantitative analyses of the relative proportions of the various oligomeric series, it seems appropriate to illustrate the complexity of the secretion's composition, as is nicely reflected by the HPLC-MS chromatograms shown in Figures 4 and 7. For oligomers consisting of more than three or four of the building blocks **6**−**8**, the number of possible isomeric structures increases dramatically. For example, there are 52 different possible pentameric azamacrolides (**12**), representing a series of eleven homologues, some of which — as a consequence of the relative proportions of the three building blocks — are present only in very small amounts. Rearrangement

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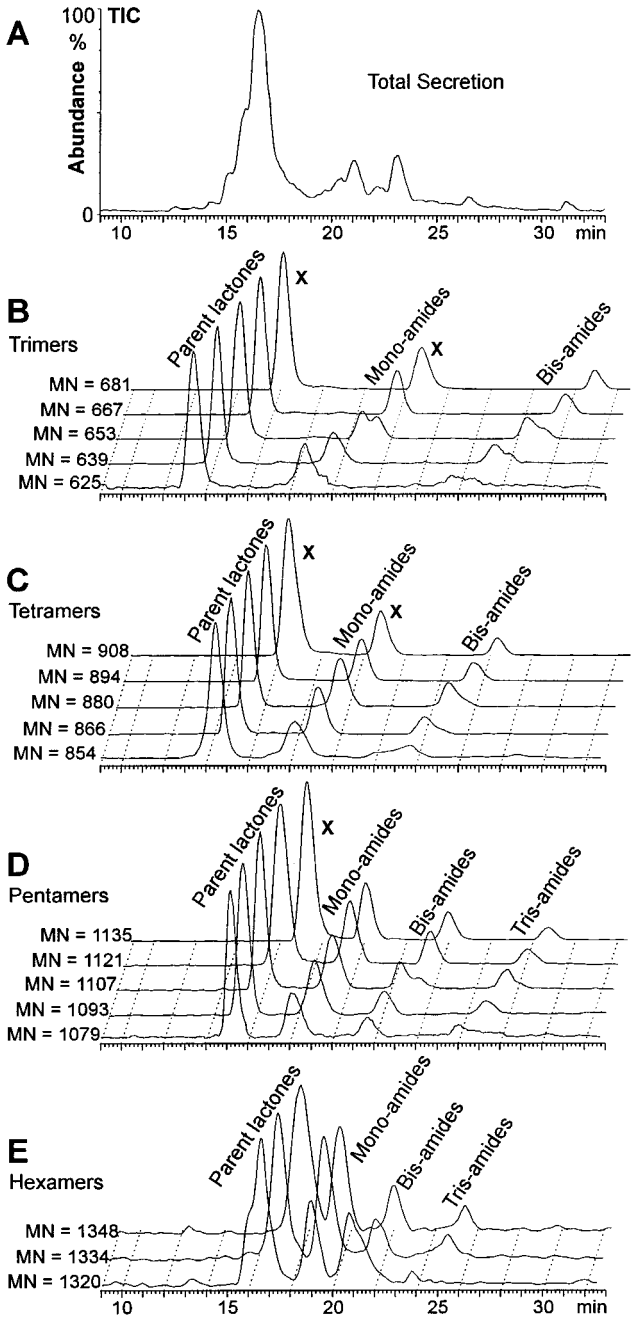

Figure 4. HPLC-MS-analysis of *E. borealis* pupal secretion: (A) total ion current (TIC) chromatogram and (B, C, D, and E) ion chromatograms for the pseudomolecular ions $(M + H)^+$ of each of five homologous trimers, tetramers**,** pentamers, and three homologous hexamers, respectively (MN = nominal molecular mass). For each displayed ion chromatogram, the largest peak has been normalized to 100%. "X" denotes peaks representing components isolated by preparative HPLC.[6]

of one or more ester linkages in these 52 pentamers then leads to more than 200 additional components. Moreover, the family of hexameric azamacrolides (**13**) alone consists of 129 compounds, representing a series of thirteen homologues, which can give rise to more than 1000 lactam isomers. Much higher numbers would be obtained from a detailed consideration of macrocycles with more than six units. Each of the different isomers of a particular species may have different HPLC retention times, as shown in Figure 4D for the all-lactonic pentamer PAML **1135** and its lactam isomers, and in Figure 7B for the corresponding peracetylated derivatives. For larger

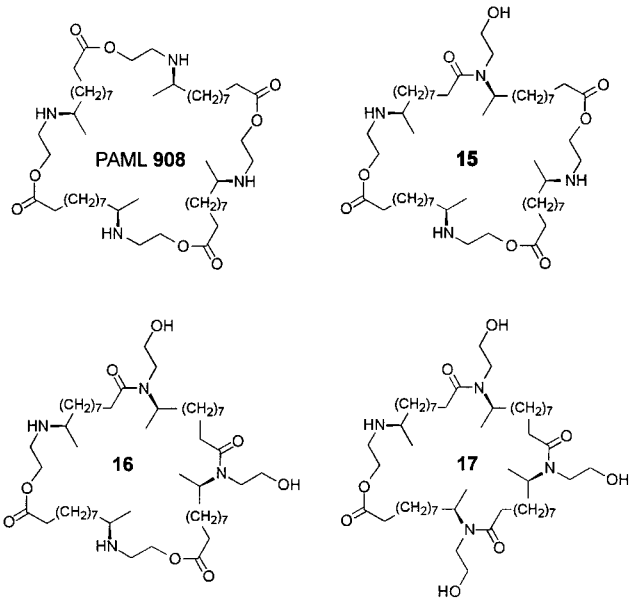

Figure 5. PAML **908** and three of its isomers, **15**−**17**, showing one to three lactam linkages, which result from intramolecular *O*-to-*N* acyl migration.

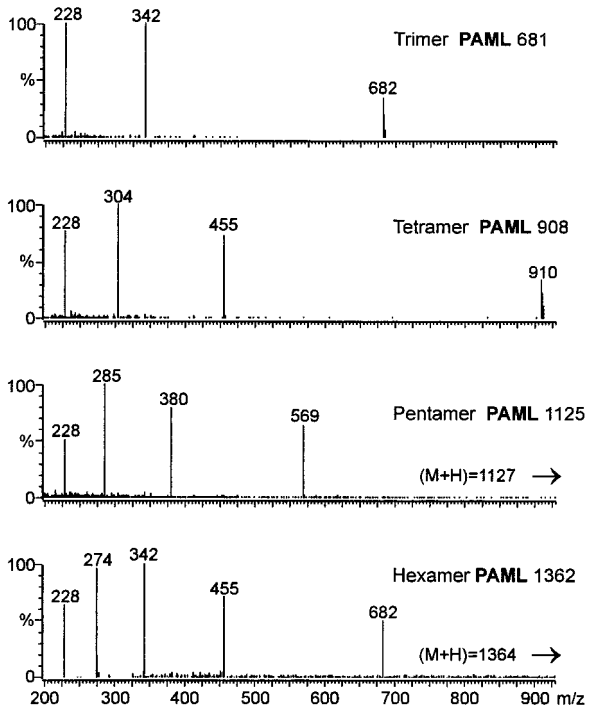

Figure 6. Positive-ion electrospray mass spectra of prominent polyazamacrolides.

oligomers, such as the nonamer PAML **2043**, the number of possible amide isomers as well as the fraction of rearranged macrocycles with one or more amide linkages is significantly higher, and the peracetylated derivatives are no longer separated by HPLC (Figure 7D). Compared to the huge separations of the corresponding underivatized macrocycles, the differences in the retention times of the various isomers of the peracetylated PAML's are generally small, facilitating a cumulative quantification of the PAML's and their lactam isomers (compare, for example, Figures 4D and 7B). Therefore, quantitative analyses of the relative proportions of the various oligomeric series and of the relative proportions of the homologues within each series were based entirely on HPLC-MS data obtained from the peracetylated secretion.

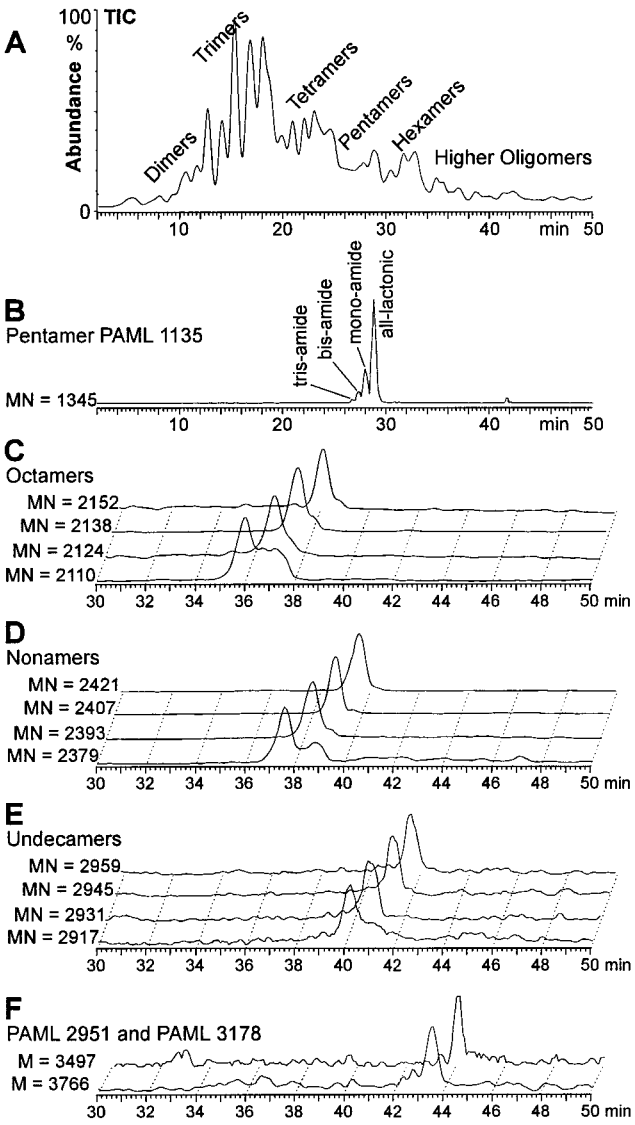

Figure 7. HPLC-MS-analysis of peracetylated *E. borealis* pupal secretion: (A) total ion current (TIC) chromatogram; (B) ion chromatogram for the pseudomolecular ion $(M + Na)^+$ of the most abundant pentamer; (C, D, and E) ion chromatograms for the pseudomolecular ions $(M + 2Na)^{2+}$ of each four homologous octamers, nonamers, and undecamers; and (F) ion chromatograms for the pseudomolecular ions $(M + 3Na)^{3+}$ of the 13-mer PAML **2951** and the 14-mer PAML **3178** (MN = nominal molecular mass). For each of the displayed ion chromatograms, the largest peak is normalized to 100%.

Due to the large number of isomers of PAML's incorporating more than one of the smaller building blocks **6** and **7**, analysis of the relative proportions of homologues within each series of oligomers was generally limited to the first two homologues in each series: the oligomer entirely derived from building block **8** and the oligomer incorporating **8** along with one unit of **7**. The relative proportions of these two homologues in each series closely match the pattern one would expect for statistical oligomerization of **7** and **8** (Figure 8A). Accordingly, the distribution of homologues becomes increasingly broad for higher oligomers, as shown in Figure 8B for the homologous series of trimers and undecamers. These data support the supposition that the three building blocks are incorporated into the macrocycles in a random fashion.

To determine the relative proportions of the different oligomeric series in the secretion, a calibration curve for the mass spectrometric response of PAML's derived from two to seven

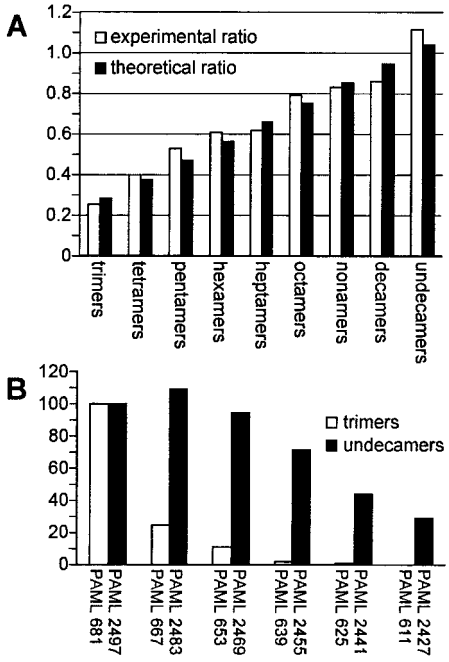

Figure 8. Quantitative analyses of the relative proportions of oligomers in each series: (A) mass spectrometric ratio of the homologue derived entirely from **8** and the homologue derived from **8** and one unit of **7**, compared with the ratio expected for random incorporation of **8** and **7** and (B) relative proportions in the series of homologous trimers and undecamers. For both the trimeric and undecameric series, the column representing the oligomer entirely derived from **8** was normalized to 100%.

units of **8** was constructed, using peracetylated samples of synthetic PAML **454**, PAML **681**, PAML **908**, PAML **1135**, PAML **1362**, and PAML **1589**.[10] The estimated relative amounts determined for each series of oligomers are given in Table 1.

Open-Chain Oligomers

With regard to the specificity of the biosynthesis of the PAML's, we thought it interesting to analyze the pupal secretion for the presence of any open-chain oligomers. From synthetic linear oligomers of three, four, five, six, and eleven units of 10-(2-hydroxyethylamino)undecanoic acid (**8**),[10] we prepared samples of the corresponding peracetylated methyl esters. These synthetic linear oligomers were used to establish a grid of HPLC retention times for detection of open-chain oligomers in the similarly derivatized pupal secretion, which led to the identification of small amounts of open-chain oligomers corresponding to the various series of cyclic oligomers (Figure 9). As shown in Figure 9C, open-chain oligomers derived from six or fewer units of the three building blocks represent only a small fraction of the related cyclic oligomers. For example, the linear trimer derived from three units of **8** occurs at a concentration less than 0.5% that of the corresponding macrocycle, PAML **681**. In contrast, for oligomers consisting of more than nine units, open-chain oligomers are present at concentrations similar to those of the corresponding macrocycles. While the small amounts of open-chain oligomers with less than six units might result from the partial hydrolysis of originally produced macrocycles either before or during derivatization,[13] it appears that for oligomers with ten or more units the efficiency of the cyclization step in the biosynthesis of the PAML's significantly decreases. In any

(13) Acetylation of samples of the synthetic PAML's resulted in formation of trace amounts of open-chain oligomers; however, the amounts detected are much smaller than those found in the peracetylated secretion.

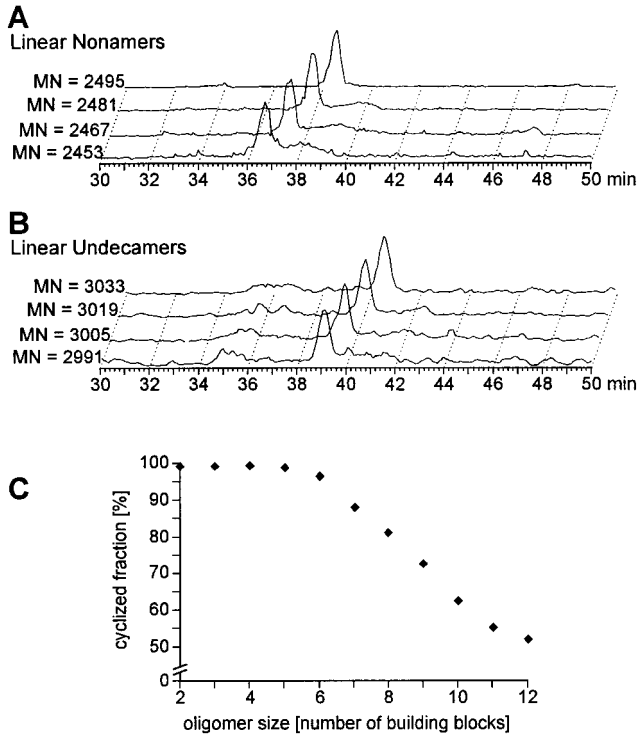

Figure 9. HPLC-MS analysis of peracetylated *E. borealis* pupal secretion: ion chromatograms for the pseudomolecular ions $(M + 2Na)^{2+}$ of each of four homologous, open-chain nonamers (A) and undecamers (B) (peracetylated and methylated), MN = nominal molecular mass, and (C) fraction of cyclized oligomer for the oligomers derived from 2 through 12 units of **8**.

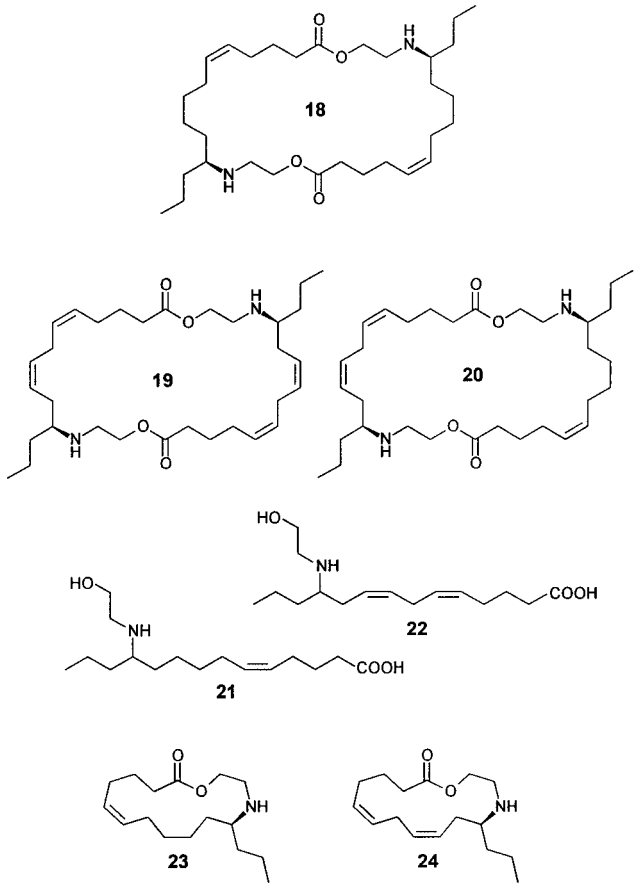

Figure 10. Macrocyclic alkaloids from *S. 24-punctata* (**18**−**20**) and *E. varivestis* (**23**, **24**) and their common building blocks (**21** and **22**).

case, open-chain oligomers of all molecular masses taken together represent only a very small fraction (about 1.0%) of the total secretion.

Discussion

In summary, the *Epilachna borealis* pupal secretion consists largely of a combinatorial library of macrocyclic polyamines with very large ring sizes, corresponding to the set of bis- to hepta-lactones **9**−**14** (Figure 3) accompanied by their various lactam isomers and smaller amounts of higher oligomers. Judging from the quantitative distribution of homologues in each oligomeric series, it appears that the three building blocks, **6**−**8**, are incorporated into these oligomers in random fashion. To our knowledge, the range of ring sizes displayed by the PAML's is unparalleled by any other group of secondary metabolites, and is reminiscent of the large cyclic oligopeptides such as the conotoxins[14] or cyclic oligosaccharides such as the cyclodextrins.[15]

Despite the structural diversity of the natural material, it is intriguing that fresh secretion from *E. borealis* does not contain detectable amounts of the monomeric azamacrolides, and that open chain oligomers are present only at very low concentrations. Consequently, the oligomerization of the building blocks **6**−**8** appears to be a well-controlled process. That the degree of oligomerization can indeed be carefully regulated is supported by our analyses of the pupal secretions of two related ladybird beetle species, *Epilachna varivestis* and *Subcoccinella 24-punctata*. In *S. 24-punctata*, the analogous secretion consists largely of the three PAML's **18**−**20**, which correspond to the three possible dimers of the two unsaturated (ω-3)-(2-hydroxyethylamino)acids, **21** and **22** (Figure 10).[16] In *E. varivestis*, the major components are the two azamacrolides epilachnene (**23**) and epilachnadiene (**24**), which are simply the monomeric cyclization products of the same two precursors, **21** and **22**.[5] Comparing the pupal secretion of *S. 24-punctata* with that of *E. varivestis*, it is surprising that, although both species use the same two (ω-3)-(2-hydroxyethylamino)acids **21** and **22** as building blocks, the alkaloid mixtures produced by each species have no overlapping constituents. The two cyclic monomers epilachnene (**23**) and epilachnadiene (**24**) are entirely absent from the alkaloid mixture of *S. 24-punctata*, and not even traces of the dimers **18**−**20** are present in the *E. varivestis* secretion.[16] Whereas both *E. varivestis* and *S. 24-punctata* each produce almost exclusively one type of macrocycle from their set of building blocks **21** and **22**, *E. borealis* utilizes a set of similar building blocks (**6**−**8**) to produce a large library of macrocycles. Thus, the oligomerization and cyclization of the fatty acid-derived building blocks **6**−**8**, **21**, and **22** is species specific and carefully controlled.

The occurrence of the oligomeric polyazamacrocycles in the defensive secretions of ladybird beetle pupae parallels the production of oligomeric azaphenalene and indolizidine alkaloids by several other ladybird beetle species and by ants from the myrmicine family. In all of these examples, relatively simple building blocks derived from the acetate pool are oligomerized yielding mixtures of structurally more complex alkaloids. The

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oligomerization of these simple components could conceivably represent a specific adaptation optimizing deterrency against particular predators, as in the case of ladybird beetles, or enhancing the efficacy as a poison to overcome prey, as in case of the predacious myrmicine ants. Producing a combinatorial library of several hundred macrocyclic polyamines, the pupae of *Epilachna borealis* have clearly exploited this strategy to its utmost extent.

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Supporting Information Available: Experimental procedures for obtaining secretion and derivatization, as well as a detailed description of the conditions used for HPLC-MS (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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