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15. "Azido"-UTP is 5-(*p*-azidotetrafluoro)benzamido-allil-uridine triphosphate. The complete synthesis of "azido"-UTP will be published elsewhere. 4-thio-UTP was synthesized from 4-thio-UMP (Sigma) as described (*14*). 5-Iodo-2′-deoxyuridine containing DNA oligonucleotide was custom synthesized (Oligos Etc., Wilsonville, OR).
16. Walking reactions were as described (*2*), except that several positions (+12, +15, +17, +39, +40, +41, +42) in the transcript were radiolabeled with [^{32}P]cytosine monophosphate and [^{32}P]guanosine monophosphate, the cross-linking with 4-thio-UMP was induced by UV irradiation at 365 nm as in (*14*), the cross-linking with "azido"-UMP was induced by UV irradiation at 308 nm for 5 min, and the cross-linking with 5-iodo-2′-deoxyuridine was performed as in (*2*). The template used was the T7A1 promoter fragment (153-pb), obtained by polymerase chain reaction with the following transcribed sequence: ATCGAGAGGGACACGGCGAATAGCCATCCCAATCGAACAGGCCTGCTGGTAATCGCAGGCCTGGAGACTTGGATCCCCGGGTA.
17. We are grateful to O. Bereschenko for help with the figures, A. Mustaev for advice on cross-linking techniques, and Sergei Borukhov for GreB. This work was supported by NIH grant GM49242 (A.G.) and departmental funds (E.N). E.A. and M.K. are in the graduate programs of the Institute of Molecular Genetics, Russian Academy of Sciences, and the Limnological Institute (Irkutsk, Russia), respectively.

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Combinatorial Chemistry in Insects: A Library of Defensive Macrocyclic Polyamines

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The pupal defensive secretion of the coccinellid beetle *Epilachna borealis* is composed principally of a combinatorial library of macrocyclic polyamines. These compounds constitute a previously unrecognized family of natural products, characterized by extremely large-ring lactonic structures derived from a small set of (2-hydroxyethylamino)alkanoic acids. The combinatorial assembly of these simple building blocks generates a high degree of structural diversity, which is further increased by slow, spontaneous intramolecular rearrangement of the macrocycles.

Insect pupae, given that they cannot crawl, run, or fly, should be vulnerable to predation. However, many benefit from concealment and camouflage or from mechanical means of defense (*1*), and there are a few documented cases of pupae that are protected chemically. Pupae of coccinellid beetles (*2*) of the genus *Epilachna* bear a dense coating of glandular hairs that secrete oily droplets deterrent to insects (Fig. 1) (*3*, *4*). In *E. varivestis* (the Mexican bean beetle), the active principles of this secretion are azamacrolides, novel lactones with a single nitrogen atom incorporated into a ring of 13 to 16 members (Fig. 1, compound **1**) (*4*). Gas chromatographic analyses of the pupal secretion of the squash beetle, *E. borealis*, have indicated only the presence of vitamin E acetate and other tocopherol derivatives (*4*, *5*). However, in tests with ants, these compounds proved to be essentially inactive, whereas the secretion itself was potently deterrent. To find and identify the active components in the *E. borealis* secretion, we therefore adopted a more general analytical approach.

Any analytical method with an initial chromatographic separation is likely to discriminate against some classes of components and to favor others. In studying a biological extract of mostly unknown composition, therefore, it is expedient to begin with a direct nuclear magnetic resonance (NMR) spectroscopic investigation of the total, unfractionated natural sample. Following this principle, we carried out NMR spectroscopic studies of freshly obtained *E. borealis* secretion (*6*). One- and two-dimensional ^{1}H NMR experiments revealed that the tocopheryl acetates account for only a relatively small percentage of the beetles' total secretion (~20%), whereas the major components represented a group of previously undetected compounds. By analysis of (^{1}H,^{1}H) exclusive correlation spectroscopy (*7*), (^{13}C,^{1}H) gradient-enhanced heteronuclear single-quantum coherence (*8*), and (^{13}C,^{1}H) gradient-enhanced heteronuclear multiple bond correlation spectra of the mixture, these components were shown to be esters and amides derived from the carboxyl- and the 2-hydroxyethylamino moieties of several (ω-1)-(2-hydroxyethylamino)alkanoic acids. To determine the chain lengths of these acids, we carried out an alkaline hydrolysis of the crude secretion, followed by methylation with diazomethane and trifluoroacetylation with trifluoroacetic acid anhydride. This procedure afforded a mixture of N,O-bis–trifluoroacetylated methyl esters of three homologous (ω-1)-(2-hydroxyethylamino)alkanoic acids, **2** to **4**, which could be analyzed by gas chromatography–mass spectrometry (GC-MS) (*9*). The major component (~90%) of the alkaline hydrolysate, 10-(2-hydroxyethylamino)undecanoic acid (**4**) was isolated by column chromatography and characterized by its ^{1}H and ^{13}C NMR spectra (*10*).

Whether the newly detected components are simply intramolecular lactones and lactams, that is, azamacrolides or their lactam isomers, or whether they each incorporate more than one acid moiety could not be determined from NMR spectra of the crude mixture alone. However, because GC-MS analysis of the crude secretion did not reveal any volatile components other than the already described tocopheryl acetates, the new *E. borealis* secretion components appeared to be higher molecular weight cyclic oligomers combining several (ω-1)-(2-hydroxyethylamino)alkanoic acid (**2** to **4**) units. This hypothesis was corroborated by high-pressure liquid chromatography (HPLC) analyses with a mass spectrometric detector, which revealed the secretion to contain a highly diverse mixture of macrocyclic polyamines (Fig. 2) (*11*). The major components are series of homologous trimers, tetramers, and pentamers of the three acids **2** to **4**, along with smaller quantities of dimers, hexamers, and heptamers (*12*). Furthermore, the secretion contains several isomers of each oligomer. Using repeated preparative HPLC fractionation, we isolated the most abundant trimeric, tetrameric, and pentameric components of the earliest eluting (Fig. 2) series of compounds (*13*). One- and two-dimensional ^{1}H NMR spectroscopic analyses (*14*) showed these components to be the symmetric macrocyclic lactones **6**, **7**, and **8** derived from three, four, or five units, respectively, of 10-(2-hydroxyethylamino)undecanoic acid (**4**) (Fig. 1). Thus, the earliest eluting, most abundant isomers of the oligomers appear to repre-

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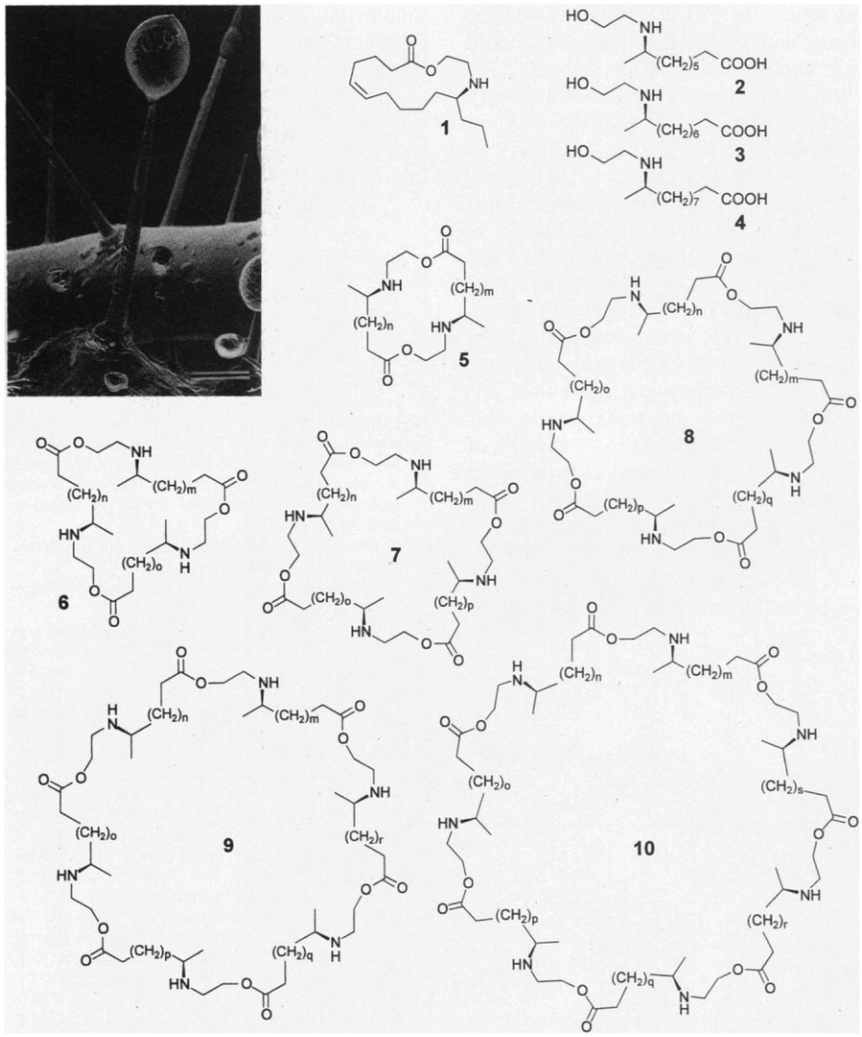

Fig. 1. (Inset) Detail of the surface of an *E. borealis* pupa, showing glandular hairs (amid nonglandular bristles; scale bar, 50 μm). Structures of epilachnene (**1**), the chief azamacrolide in the pupal defensive secretion of *E. varivestis*, and of the (ω-1)-(2-hydroxyethylamino)alkanoic acids **2**, **3**, and **4**, the building blocks of the PAMLs **5** to **10** in *E. borealis*. In these formulas, each of the variables *m* ... *s* can have the values 5, 6, or 7.

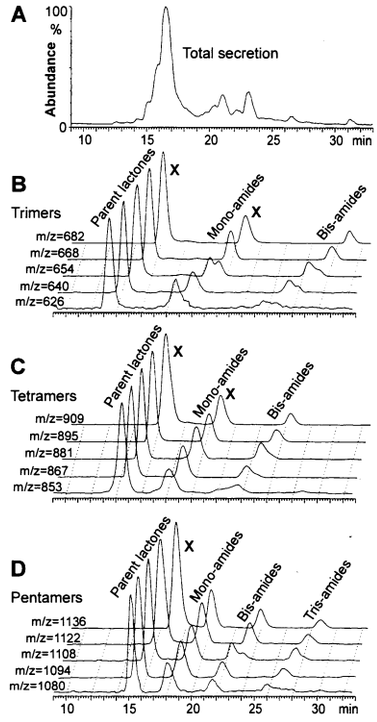

Fig. 2. HPLC-MS analysis of *E. borealis* pupal secretion, taken from 4-day-old pupae. (**A**) Total ion current chromatogram. (**B** to **D**) Ion chromatograms for the pseudomolecular ions $(M + H)^+$ of each of five homologous series of trimers (B), tetramers (C), and pentamers (D). For each of the displayed ion chromatograms, the largest peak is normalized to 100%. X, peaks representing components that were isolated by preparative HPLC (*11*). m/z is the mass-to-charge ratio.

sent the macrocyclic lactones **5** to **10** ["polyazamacrolides" (PAMLs)] generated by a nonselective oligomerization of the three building blocks, **2**, **3**, and **4**.

To confirm our structural assignments, we carried out a total synthesis of the most abundant trimer, PAML **681** (Fig. 3A). Starting from L-alanine (**11**), a linear 10-(2-hydroxyethylamino)undecanoic acid trimer (**14**) was prepared and cyclized. The final product was indistinguishable from the purified natural material in its NMR and HPLC-MS properties.

As shown in Fig. 2, HPLC-MS analyses of the pupal secretion indicated the presence of several later eluting isomers of each of the PAMLs, **5** to **10**. The members of any given group of isomers show strikingly different mass spectra. Under our experimental conditions, the trimeric, tetrameric, and pentameric PAMLs, **6**, **7**, and **8**, produced multiply charged cations with up to three, four, or five charges, respectively, corresponding to the number of basic nitrogen atoms in each compound. The later eluting isomers of each of these PAMLs did not achieve this degree of protonation (Fig. 4). The second series of trimers, for example, produced mostly singly and doubly charged pseudomolecular ions, $(M + H)^+$ and $(M + 2H)^{2+}$, whereas the third series of trimers showed predominantly singly charged ions, $(M + H)^+$ (M is the molecular weight of the molecule). This behavior is nicely rationalized on the basis of additional structural data, obtained from NMR spectroscopic studies of individual components.

The most abundant components of the later eluting, second series of trimers and tetramers (Fig. 2) were isolated by preparative HPLC (*13*). NMR spectroscopic analyses (*15*) showed the isolated trimer to consist of three units of the hydroxy amino acid **4**, which are connected by two ester linkages plus one amide linkage (structure **15**, Fig. 4). Because the monoamide **15** has one basic center less than the all-lactonic isomer PAML **681**, it shows less highly protonated pseudomolecular ions in its electrospray mass spectra (Fig. 4). NMR and mass spectroscopic studies on the isolated tetramer yielded analogous results consistent with structure **17**.

Thus, their mass spectroscopic properties characterize the later eluting isomers of the PAMLs **5** to **10** as compounds with one or more amide linkages and a correspondingly reduced number of ester linkages (Fig. 2). Using isolated samples of the most abundant entirely lactonic trimer (PAML **681**), tetramer (PAML **908**), and pentamer (PAML **1135**), we showed that the corresponding isomeric compounds with one or more amide linkages form spontaneously (*16*). Accordingly, the extent to which amides are observed in the freshly obtained native secretion depends substantially on the age of the pupae. O-Acylated α-amino alcohols, for example O-acylated serine moieties in peptides (*17*), are well known to

undergo intramolecular O-to-N acyl migration easily. It seems likely, therefore, that the presence of macrocyclic amides in the native secretion results from the intramolecular rearrangement of the initially produced PAMLs **5** to **10**.

We have shown that the *E. borealis* pupal secretion consists largely of a combinatorial library of macrocyclic polyamines with very large ring sizes (up to 98 members), corresponding to the set of the bis- to heptalactones **5** to **10** (Fig. 1), accompanied by their various amide isomers (*18*, *19*). Judging from the quantitative distribution of homologs in each oligomeric series (Fig. 3B), it appears that the three building blocks, **2**, **3**, and **4**, are incorporated into these oligomers in random fashion.

Why has this beetle prepared this library of macrocycles? Does the variety of components provide for increased efficacy of the secretion against predators? Is the biosynthesis carried out in such a way that it is necessarily indiscriminate with respect to which acid homologs are incorporated and what the final ring size will be? What is the mode of action of this highly lipophilic series of relatively strong bases, and what is the relevance of incorporating vitamin E derivatives into the pupal secretion? Although many such questions remain, this study has revealed insects to be capable of producing an unprecedented array of novel macrocyclic structures. This library of alkaloids is based on the oligomerization of only a few homologous building blocks, which, like many ladybird alkaloids (*2*), seem to be derived from the amination of simple fatty acids. Ladybird beetles must be counted among the pioneers of combinatorial chemistry.

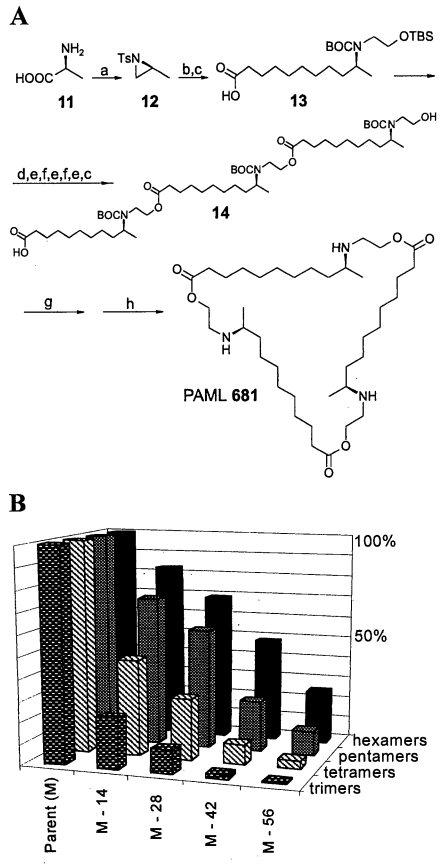

Fig. 3. (**A**) Synthesis of PAML **681**. a: 1, $LiBH_4$, trimethylsilyl chloride, and THF (*20*); 2, TsCl, pyridine, and CH_2Cl_2; 3, K_2CO_3 and acetone. b: 1, bis(3-butenyl)magnesium, CuI, and Et_2O; 2, NaH and $Br(CH_2)_2OTBDMS$; 3, $C_{10}H_7Na$ and 1,2-dimethoxyethane; 4, Boc_2O and THF; 5, O_3 and CH_2Cl_2; 6, KHMDS, $PPh_3(CH_2)_4CO_2H$, and THF. c: H_2, Pd/C, and MeOH. d: BnOH, dicyclohexylcarbodiimide (DCC), DMAP, and CH_2Cl_2. e: Bu_4NF and THF. f: **13**, DCC, DMAP, and CH_2Cl_2. g: Mukaiyama salt, Et_3N, and CH_3CN, high dilution (*21*). h: CF_3CO_2H. Ts, tosyl; Et, ethyl; OTBDMS, ±-butyldimethylsilyloxy; Boc, butoxycarbonyl; KHMOS, potassium bis(trimethylsilyl)amide; Ph, phenyl; Me, methyl; Bn, benzyl; DMAP, 4-dimethylaminopyridine; and Bu, butyl. (**B**) Relative abundances (in percent) of five homologous trimers (**6**), tetramers (**7**), pentamers (**8**), and hexamers (**9**). For each series of homologs, the column representing the parent component (the oligomer made up entirely from **4**) is normalized to 100%.

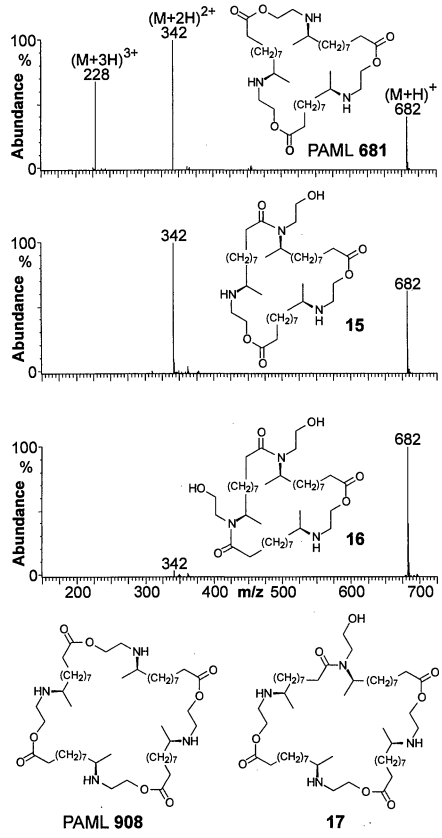

Fig. 4. Positive ion electrospray mass spectra of three isomers of the most abundant trimer (nominal molecular weight M = 681). The spectrum of the trislactone PAML **681** shows the pseudomolecular ions m/z = 228 for $(M + 3H)^{3+}$, m/z = 342 for $(M + 2H)^{2+}$, and m/z = 682 for $(M + H)^{+}$. The spectrum of monoamide **15** shows only m/z = 342 for $(M + 2H)^{2+}$ and m/z = 682 for $(M + H)^{+}$. The spectrum of the bisamide **16** shows predominantly m/z = 682. The structures of PAML **908** and PAML **17** are also shown.

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6. *Epilachna borealis* pupae (3 to 5 days old) were washed with 0.2 ml of dichloromethane. The combined washings of 30 to 60 pupae were evaporated in vacuo. Subsequently, the oily, colorless residue (about 40 μg per pupa) was directly submitted to NMR analysis.
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9. A Hewlett-Packard 5890 II gas chromatograph was linked to a Hewlett-Packard 5970 mass selective detector. A 30-m fused silica column coated with DB5-MS (0.25-μm film) with an inside diameter of 0.25 mm was used. The conditions were 3 min at 80°C and then increased 10°C/min to 290°C. The relative abundances (total ion current) of the derivatives of the (ω-1)-(2-hydroxyethylamino)alkanoic acids **2**, **3**, and **4** were 1.5:8.5:90, respectively. The absolute configuration of the asymmetric center in **2**, **3**, and **4** was determined to be *R* with more than 99% enantiomeric excess [F. C. Schröder, J. J. Farmer, S. R. Smedley, T. Eisner, J. Meinwald, unpublished results].
10. 10-(2-Hydroxyethylamino)undecanoic acid (**4**): ^1H NMR {500 MHz, CD_3OD, δ [parts per million (ppm)]}: 1.31 (d, J = 6.5 Hz, 3H, 11-H), 1.33 to 1.49 (m, 10H), 1.50 to 1.65 (m, 3H), 1.74 to 1.81 (m, 1H), 2.28 (t, J = 7.2 Hz, 2H, 2-H), 3.08 to 3.16 (m, 2H, CH_2-N), 3.22 to 3.30 (m, 1H, 10-H), 3.79 (t, J = 7.0 Hz, 2H, CH_2-O). ^{13}C NMR [126 MHz, CD_3OD, δ (ppm)]: 16.0, 25.9, 29.9, 30.0, 30.0, 30.1, 30.2, 33.7, 34.7, 47.4, 55.4, 57.8, 177.7.
11. A Hewlett-Packard 1090 II pump was linked to a Micromass Quattro I mass spectrometer operated in positive ion electrospray mode. The HPLC column was a 250 mm × 46 mm Inertsil 5-μ ODS-3 (Metachem). The flow was 0.7 ml/min. The solvent gradient system was as follows: from a mixture of 95% water, 4.5% acetonitrile, 0.4% tetrahydrofuran (THF), and 0.1% formic acid to 60% water, 22% acetonitrile, 17.9% THF, and 0.1% formic acid over a period of 32 min.
12. The estimated relative amounts of each series of oligomers, based on the relative abundance of corresponding pseudomolecular ions obtained by HPLC-MS (*11*), are 1% dimers, 47% trimers, 37% tetramers, 11% pentamers, 3% hexamers, and 1% heptamers.
13. HPLC conditions used for isolation of PAML **681** (300 μg), PAML **908** (190 μg), and PAML **1135** (110 μg) were as follows: the HPLC system and column were as in (*11*); the flow was 1.1 ml/min. The solvent gradient system was as follows: from a mixture of 95% water, 4% acetonitrile, 0.94% THF, and 0.06% formic acid to 84% water, 9.5% acetonitrile, 6.44% THF, and 0.06% formic acid over a period of 45 min. The monoamide trimer **15** (120 μg) and the corresponding monoamide tetramer **17** (90 μg) were isolated with the HPLC conditions given in (*11*). The compounds were isolated in 70 to 90% purity.
14. ^1H NMR data of PAML **681** [500 MHz, C_6D_6, δ (ppm)]: 0.97 (d, J = 6.4 Hz, 9H, 11-H), 1.19 to 1.41 (m, 36H, 4, 5, 6, 7, 8, and 9-H), 1.63 (quin., J = 7.1 Hz, 6H, 3-H), 2.20 (t, J = 7.4 Hz, 6H, 2-H), 2.49 to 2.56 (m, 3H, 10-H), 2.63 (ddd, $J_{N\text{-}CHaHb}$ = 12.7 Hz, $J_{N\text{-}CHaHb,O\text{-}CHaHb}$ = 6.8 Hz, $J_{N\text{-}CHaHb,O\text{-}CHaHb}$ = 4.4 Hz,

3H, N-C$\underline{H}_a$H$_b$), 2.72 (ddd, $J_{N\text{-}CHaHb,O\text{-}C\underline{H}aHb}$ = 4.3 Hz, $J_{N\text{-}CHa\underline{H}b,O\text{-}CHa\underline{H}b}$ = 6.3 Hz, 3H, N-CH$_a\underline{H}_b$), 4.14 (ddd, $J_{O\text{-}C\underline{H}aHb}$ = 11.1 Hz, 3H, O-C$\underline{H}_a$H$_b$), 4.20 (ddd, 3H, O-CH$_a\underline{H}_b$). ^{1}H NMR data of PAML **908** [500 MHz, C_6D_6, δ (ppm)]: 0.98 (d, J = 6.2 Hz, 12H, 11-H), 1.19 to 1.43 (m, 48H, 4, 5, 6, 7, 8, and 9-H), 1.60 to 1.67 (m, 8H, 3-H), 2.22 (t, J = 7.4 Hz, 8H, 2-H), 2.50 to 2.57 (m, 4H, 10-H), 2.65 (ddd, $J_{N\text{-}C\underline{H}aHb}$ = 12.6 Hz, $J_{N\text{-}C\underline{H}aHb,O\text{-}C\underline{H}aHb}$ = 6.7 Hz, $J_{N\text{-}C\underline{H}aHb,O\text{-}CHa\underline{H}b}$ = 4.5 Hz, 4H, N-C$\underline{H}_a$H$_b$), 2.74 (ddd, $J_{N\text{-}CHa\underline{H}b,O\text{-}C\underline{H}aHb}$ = 4.6 Hz, $J_{N\text{-}CHa\underline{H}b,O\text{-}CHa\underline{H}b}$ = 6.3 Hz, 4H, N-CH$_a\underline{H}_b$), 4.14 to 4.19 (m, 4H, O-C$\underline{H}_a$H$_b$), 4.19 to 4.23 (m, 3H, O-CH$_a\underline{H}_b$). The ^{1}H NMR chemical shift values of PAML **1135** closely resemble those given for PAML **908**.

15. ^{1}H NMR data of the monoamide trimer **15** [500 MHz, C_6D_6, δ (ppm)]: 0.71 (d, J = 6.4 Hz, 3H, 11-H of N-acylated unit), 0.95 (m, 1H, 9-H$_a$ of N-acylated unit), 0.97 (d, J = 6.4 Hz, 6H, 11-H of O-acylated unit), 1.10 (m, 1H, 9-H$_b$ of N-acylated unit), 1.18 to 1.52 (m, 34H, 4, 5, 6, 7, and 8-H of all three units and 9-H of O-acylated units), 1.57 to 1.83 (m, 6H, 3-H of all three units), 2.09 to 2.16 (m, 1H, 2-H$_a$ of N-acyl unit), 2.16 to 2.24 (m, 1H, 2-H$_b$ of N-acyl unit), 2.19 to 2.24 (m, 4H, 2-H of O-acyl units), 2.48 to 2.55 (m, 2H, 10-H of O-acylated units), 2.58 to 2.64 (ddd-like m, 2H, N-C$\underline{H}_a$H$_b$ of O-acylated units), 2.68 to 2.75 (m, 2H, *N*-CH$_a\underline{H}_b$ of O-acylated units), 3.15 (dt, $J_{N\text{-}C\underline{H}aHb,N\text{-}CHa\underline{H}b}$ = 14.1 Hz, $J_{N\text{-}C\underline{H}aHb,O\text{-}C\underline{H}aHb}$ = 5.5 Hz, 1H, N-C$\underline{H}_a$H$_b$ of N-acylated unit), 3.32 (dt, $J_{N\text{-}C\underline{H}aHb,N\text{-}CHa\underline{H}b}$ = 14.1 Hz, $J_{N\text{-}CHa\underline{H}b,O\text{-}CHa\underline{H}b}$ = 5.5 Hz, 1H, N-CH$_a\underline{H}_b$ of N-acylated unit), 3.39 to 3.47 (m, 1H, 10-H of N-acylated unit), 3.72 to 3.76 (m, 2H, C$\underline{H}_2$-OH), 4.11 to 4.17 (m, 2H, O-C$\underline{H}_a$H$_b$ of O-acylated units), 4.18 to 4.24 (m, 2H, O-CH$_a\underline{H}_b$ of O-acylated units).

16. To estimate the rate of isomerization, we stored pure, neat samples of PAML **681**, PAML **908**, and PAML **1135** at 25°C for 2 days. Subsequent analyses by HPLC-MS revealed 7%, 10%, and 12% of monoamide (relative percentage of the total ion current) for the trimer, tetramer, and pentamer, respectively.

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