

|                                          |                                             |
|------------------------------------------|---------------------------------------------|
| SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO |                                             |
| RAD: 13-16116- -6                        | FECHA: 2015-05-16 23:32:39                  |
| DEP: 2020 DIRECCION DE NUEVAS CREACIONES | EVE: 1 REGISTRO/DEPOSITO/CONCESION/DEPOSITO |
| TRA: 2 PATENTES DE INVENCION             | FOLIOS: 6                                   |
| ACT: 430 REQUERSOLICITA                  |                                             |

DIRECCIÓN DE NUEVAS CREACIONES

2020  
Bogotá, D.C.,

Abogado(a)/Señor(a)  
JULIO CESAR PADILLA HERRERA

Asunto:      Radicación:      13-16116- -6  
                 Trámite:              2  
                 Evento:              1  
                 Actuación:            430  
                 Oficio:                    5377  
                 Folios:                    6

|                                     |   |            |  |                               |  |
|-------------------------------------|---|------------|--|-------------------------------|--|
| Patente de Invención                | X |            |  | Patente de Modelo de Utilidad |  |
| Vía Nacional                        | X |            |  |                               |  |
| Vía PCT (Fase Nacional)             |   | Capítulo I |  | Capítulo II                   |  |
| No. Solicitud Internacional         |   |            |  |                               |  |
| Fecha de Presentación Internacional |   |            |  |                               |  |

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

|                   |                                  |               |
|-------------------|----------------------------------|---------------|
| Descripción:      | Radicado N° 13-016116-00000-0000 | 24/Enero/2013 |
| Reivindicaciones: | Radicado N° 13-016116-00000-0000 | 24/Enero/2013 |
| Dibujos:          | Radicado N° 13-016116-00000-0000 | 24/Enero/2013 |

2. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por la solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso se estudiarán las reivindicaciones 1 a 18 del capítulo reivindicatorio (Radicado N°. 13-016116-00000-0000).

Título de la solicitud: “PEPTIDO SINTÉTICO POLICATIONICO CON PROPIEDADES IONOFÓRICAS, ANTIMICROBIANAS ANTITUMORALES E INSECTICIDAS”.

El problema planteado en esta solicitud consiste en la necesidad de proporcionar péptidos útiles como bactericidas, fungicidas, antitumorales e insecticidas.

Para resolver este problema técnico la solicitud en estudio proporciona el péptido sintético BTM-P4, de secuencia IAPALIAVAPIAKYLATALAKWALKQGFAKLKS (SEQ ID No. 1: BTM-P4).

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A01N 37/18.

### 3. No es una invención (Art 15 D 486 literal (b))

El objeto de las reivindicaciones 1 – 3, 5 – 7, 13 y 14 no se considera una invención, de acuerdo con el Art citado, ya que la secuencia del péptido reclamado se encuentra comprendida en la secuencia de una proteína encontrada en la naturaleza en una cepa de *Bacillus thuringiensis*, tal como se demuestra en el alineamiento mostrado a continuación:

|                                                                              |        |                                   |                               |             |          |
|------------------------------------------------------------------------------|--------|-----------------------------------|-------------------------------|-------------|----------|
| delta-endotoxin [Bacillus thuringiensis]                                     |        |                                   |                               |             |          |
| Sequence ID: <a href="#">gb ADI59541.1 </a> Length: 792 Number of Matches: 1 |        |                                   |                               |             |          |
| Range 1: 28 to 60 <a href="#">GenPept</a> <a href="#">Graphics</a>           |        |                                   | ▼ Next Match ▲ Previous Match |             |          |
| Score                                                                        | Expect | Method                            | Identities                    | Positives   | Gaps     |
| 69.3 bits(168)                                                               | 3e-12  | Composition-based stats.          | 33/33(100%)                   | 33/33(100%) | 0/33(0%) |
| Query                                                                        | 1      | IAPALIAVAPIAKYLATALAKWALKQGFAKLKS | 33                            |             |          |
|                                                                              |        | IAPALIAVAPIAKYLATALAKWALKQGFAKLKS |                               |             |          |
| Sbjct                                                                        | 28     | IAPALIAVAPIAKYLATALAKWALKQGFAKLKS | 60                            |             |          |

La secuencia del gen codificante para dicha proteína se encuentra reportada en el documento D1.

### 4. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 15 – 18 no es patentable, de acuerdo con el Art 14, puesto que no hacen referencia a productos o procedimientos, y por lo tanto se considera que reclaman materia no patentable.

### 5. De la solicitud de patente

#### Reivindicaciones (Art 30 D 486, Art 22 PCT)

- 1- La reivindicación 8 no es clara, ya que al reclamar una formulación farmacéutica se debe caracterizar por medio de la identidad química (dada por nombre IUPAC, denominación común internacional o estructura) de los excipientes presentes en la misma, así como las proporciones o concentraciones de los mismos. En este sentido el término “vehículos farmacéuticamente aceptables” no es admisible puesto que se refiere a la función del componente y no a sus características estructurales. Se solicita definir la composición adecuadamente. Lo mismo es aplicable para las reivindicaciones 11 y 12.
- 2- La reivindicación 9 no es clara puesto que caracteriza la formulación reclamado en función de un resultado a alcanzar, es decir, la actividad biológica proclamada para el péptido comprendido en dicha formulación. Puesto que esta no es una característica estructural y se refiere no sólo la solución propuesta por la solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado, se solicita eliminar la referencia a dichos resultados a alcanzar y/o reemplazarlos por las características esenciales, estructurales o funcionales, que contribuyen a la obtención de dicha actividad.
- 3- La reivindicación 9 no es clara puesto que el término “cantidad farmacéuticamente efectiva” es impreciso por lo que no caracteriza de manera adecuada la formulación

reclamada, se solicita definir las cantidades, proporciones o concentraciones usando para ello un rango numérico concreto con sus respectivas unidades.

- 4- La reivindicación 10 no es clara porque caracteriza la formulación farmacéutica usando términos referidos a su forma de administración “oral, tópica, rectal o parenteral” las cuales hacen referencia a métodos de tratamiento y no son características esenciales de la formulación reclamada. Se solicita eliminar las referencias a la administración y caracterizar la formulación de acuerdo a lo descrito anteriormente.
- 5- Las reivindicaciones 11 y 12 no proporcionan características técnicas adicionales a las establecidas en la reivindicación 8, por lo que se consideran redundantes. Se sugiere modificarlas para que comprendan características técnicas adicionales o eliminarlas del capítulo reivindicatorio.

Las reivindicaciones 1 y 2 no son concisas porque el término “*derivados*” y la expresión “*presenta una homología de al menos 90% a la SEQ ID no. 1*” dan lugar a un gran número de péptidos posibles que no se encuentran sustentados en la descripción, puesto que en esta se presentan sustento para la evaluación de la actividad biológica únicamente para los péptidos de secuencia SEQ ID No. 1 que comprenden aminoácidos de isomería D y L (Págs. 19-23), por lo que no existe evidencia de que al realizar modificaciones en la secuencia reclamada la actividad se mantenga. De esta forma se sugiere restringir la solicitud a una razonable generalización del tipo de péptidos que han sido sintetizados ó probados, es decir, que se encuentran debidamente sustentados.

Se sugiere a la solicitante hacer las aclaraciones necesarias.

#### **Descripción (Art 28 D 486)**

La descripción no divulga la invención de manera suficientemente completa para que el la persona del oficio normalmente versada en la materia pueda comprender el efecto técnico reclamado puesto que no se muestra la secuencia del péptido usado como comparativos (BTM-P1 y BTM-P1d).

Se requiere a la solicitante hacer las aclaraciones necesarias.

#### **6. Determinación del Estado de la Técnica**

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de patente nacional: enero 24 de 2013 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

| Ítem | Documento                                                                                 | Título                                                                                        | Fecha de Publicación |
|------|-------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|----------------------|
| D1   | Melnikov O et al. J Mol Microbiol Biotechnol 2011;20:204–210                              | Tandem Repeats in a New Toxin Gene from Bacillus thuringiensis and in Other cry11 -Like Genes | 19/Julio/2011        |
| D2   | C. Segura et al. / Biochemical and Biophysical Research Communications 353 (2007) 908–914 | BTM-P1 polycationic peptide biological activity and 3D-dimensional structure                  | 22/Diciembre/2006    |

El documento D1<sup>1</sup> divulga la secuencia de un nuevo gen, cry11Bb2 aislado de una cepa de campo de *Bacillus thuringiensis*, clonado y expresado en *E.coli* para producir una proteína Cry11Bb1 (Pág. 204, Resumen).

El documento D2<sup>2</sup> divulga la estructura del péptido BTM-P1 y su actividad como agente antimicrobiano contra bacterias Gram positivas y Gram negativas (Pág. 908, Resumen).

El documento D3 divulga la aplicación de los aminoácidos D en el diseño de péptidos que adoptan conformaciones de plegado bien definidas (Pág. 415, introducción).

## 7. Examen de Patentabilidad (Art 14 D486)

### Nivel inventivo (Art 18 D486)

La reivindicación 4 consiste en: “Un péptido de la reivindicación 1, caracterizado porque todos los aminoácidos que lo componen presentan isomería de tipo D” (Radicado N°. 13-016116-00000-00000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

| Características esenciales                | D1                                                                                                | D2                                                                                                                                 |
|-------------------------------------------|---------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------|
| Polipéptido de secuencia                  | Endotoxina $\delta$ de <i>Bacillus thuringiensis</i> . Cry11Bb2                                   | Péptidos sintéticos correspondientes al dominio I de la toxina Cry11Bb1 (Pág. 910, tabla 1)                                        |
| IAPALIAVAPIAKYLATALAKWAL KQGFAKLKS        | Que comprende la secuencia:<br><br><sup>28</sup> IAPALIAVAPIAKYLATALAKWA LKQGFAKLKS <sup>60</sup> | BTM-P1<br><sup>35</sup> VAPIAKYLATALAKWALKQGF AKLKS <sup>60</sup><br>BTM-P8<br><sup>24</sup> IEPSIAPALIAVAPIAKYLATAL <sup>46</sup> |
| Donde todos los aminoácidos son de tipo D | No se menciona                                                                                    | No se menciona                                                                                                                     |

El documento D1 se considera el estado de la técnica cercano a la solicitud definida en la reivindicación 4 porque divulga un gen que codifica para la proteína Cry11Bb2 (accession # HM068615) (Pág. 205), la cual comprende la secuencia del péptido reclamado actualmente (Ver alineamiento en el numeral 3).

La diferencia entre el péptido reclamado por la reivindicación 4 y la materia divulgada en el documento D1 consiste en que la reivindicación 4 reclama péptidos, es decir segmentos de la proteína Cry11Bb2, conformados por aminoácidos de isomería D, mientras que el documento D1 hace referencia a la proteína completa Cry11Bb2 tal como se encuentra en la naturaleza.

El efecto logrado por la selección del péptido que comprende la secuencia de aminoácidos de la enzima Cry11Bb2 entre los aminoácidos 28-60 conformado por aminoácidos de isomería D, es su aplicación como agentes antimicrobianos.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular como la necesidad de proporcionar péptidos sintéticos útiles como antimicrobianos derivados de la proteína Cry11Bb2.

1<http://www.ncbi.nlm.nih.gov/pubmed/21778765>

2<http://www.sciencedirect.com/science/article/pii/S0006291X06027628>

Sin embargo, la selección de fragmentos de endotoxinas  $\delta$  de *Bacillus thuringiensis* para proporcionar péptidos biológicamente activos, se encuentra divulgada en el documento D2, el cual se refiere a péptidos que corresponden al dominio I de la endotoxina Cry11Bb1 que poseen actividad como antimicrobianos (Pág. 910, Tabla 1). Por otra parte la sustitución con aminoácidos de isomería D para el diseño de péptidos antimicrobianos, es materia conocida por la persona del oficio normalmente versada en la materia<sup>3,4</sup>.

En consecuencia, la persona del oficio normalmente versada en la materia, desarrollaría péptidos antimicrobianos acuerdo a lo enseñado en el documento D2, a partir de segmentos de la proteína Cry11bb2 divulgada por el documento D1, llegando así al objeto de la reivindicación 4 de la solicitud. Por lo cual la materia reclamada se considera obvia.

Puesto que las reivindicaciones 8-12 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

#### Conclusión:

Las reivindicaciones 4, 8 – 12 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

### 8. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

| Ítem | Documento                                                                               | Reivindicaciones afectadas | Requisito que afecta |
|------|-----------------------------------------------------------------------------------------|----------------------------|----------------------|
| D1   | Melnikov O et al. J Mol Microbiol Biotechnol 2011;20:204–210                            | 4, 8 – 12                  | Nivel inventivo      |
| D2   | C. Segura et al. Biochemical and Biophysical Research Communications 353 (2007) 908–914 | 4, 8 – 12                  | Nivel inventivo      |

3<http://www.nmr2.buffalo.edu/resources/edu/matr/TheUseofDAminoAcidsinPeptideDesign.pdf>

4<http://www.pnas.org/content/87/12/4761.full.pdf>

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



**JOSÉ LUIS SALAZAR LÓPEZ**  
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2015-05-19

Elaboró: Ruth Andrea Rodriguez  
Revisó: Jeny Paola Villate Becerra

| INFORME DE BÚSQUEDA DE PATENTES PARA EL No de Radicación: 13-016116                                                                                                       |                                                         |                                                                                                      |                 |                                   |                                                                  |                |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------|------------------------------------------------------------------------------------------------------|-----------------|-----------------------------------|------------------------------------------------------------------|----------------|
| CRITERIOS PARA LA BÚSQUEDA                                                                                                                                                |                                                         |                                                                                                      |                 |                                   |                                                                  |                |
| Palabras clave utilizadas                                                                                                                                                 |                                                         | D amino acid peptide<br>antimicrobial<br>BTM-P<br>Cry11Bb toxin<br>Endotoxin Bacillus thuringiensis  |                 |                                   |                                                                  |                |
| CLASIFICACIONES INTERNACIONALES UTILIZADAS PARA LA BÚSQUEDA                                                                                                               |                                                         | A01N 37/18                                                                                           |                 |                                   |                                                                  |                |
| BASES DE DATOS CONSULTADAS.                                                                                                                                               |                                                         |                                                                                                      |                 |                                   |                                                                  |                |
| NCBI                                                                                                                                                                      |                                                         | The Lens                                                                                             |                 | Spacenet                          |                                                                  | Science direct |
| INFORMES DE EXÁMENES REALIZADOS EN OTRAS OFICINAS                                                                                                                         |                                                         |                                                                                                      |                 |                                   |                                                                  |                |
| Oficina                                                                                                                                                                   | Fecha del examen o de reporte de búsqueda de documentos | Anterioridades encontradas para el examen o reportados en el examen de búsqueda(relacionarlos todos) | Reiv. afectadas | RELEVANCIA (requisito que afecta) | FUERON TENIDOS EN CUENTA PARA EL EXAMEN EN LA OFICINA COLOMBIANA |                |
|                                                                                                                                                                           |                                                         |                                                                                                      |                 |                                   | SI                                                               | NO             |
| EUROPEA                                                                                                                                                                   | NA                                                      | No se ha realizado búsqueda o examen                                                                 |                 |                                   |                                                                  |                |
| USPTO                                                                                                                                                                     | NA                                                      |                                                                                                      |                 |                                   |                                                                  |                |
| DOCUMENTOS ADICIONALES ENCONTRADOS POR EL EXAMINADOR DE ACUERDO A LOS CRITERIOS DE BÚSQUEDA.                                                                              |                                                         |                                                                                                      |                 |                                   |                                                                  |                |
| Melnikov O et al. J Mol Microbiol Biotechnol 2011;20:204–210. <i>Tandem Repeats in a New Toxin Gene from Bacillus thuringiensis and in Other cry11 -Like Genes</i>        |                                                         |                                                                                                      |                 |                                   |                                                                  |                |
| Segura et al, BTM-P1 polycationic peptide biological activity and 3D-dimensional structure. <i>Biochemical and Biophysical Research Communications</i> 353 (2007) 908–914 |                                                         |                                                                                                      |                 |                                   |                                                                  |                |
| Mahalakshmi and Balaram. <i>D amino acids: A New Frontier in Amino Acid and Protein Research, Chapter 5.9: The Use of D amino acids in peptide design.</i> Pags.415-430.  |                                                         |                                                                                                      |                 |                                   |                                                                  |                |
| Wade et al. Proc. Natl. Acad. Sci. USA, Vol. 87, pp. 4761-4765, June 1990. Biochemistry All-D amino acid-containing channel-forming antibiotic peptides                   |                                                         |                                                                                                      |                 |                                   |                                                                  |                |
| BÚSQUEDA DE SECUENCIAS                                                                                                                                                    |                                                         |                                                                                                      |                 |                                   |                                                                  |                |
| Número de depósito / patente y N° de secuencia<br>% de identidad                                                                                                          |                                                         |                                                                                                      |                 |                                   |                                                                  |                |
| SEQ ID NO (de la solicitud)                                                                                                                                               | NCBI                                                    | The Lens                                                                                             | EMBL-EBI        | DDJB                              | Genome Quest                                                     |                |
| 1                                                                                                                                                                         | GI: 298162158                                           |                                                                                                      |                 |                                   |                                                                  |                |
|                                                                                                                                                                           | 100 % de identidad                                      |                                                                                                      |                 |                                   |                                                                  |                |

## CRITERIOS DE BÚSQUEDA

delta-endotoxin [Bacillus thuringiensis]

Sequence ID: [gb|ADI59541.1|](#) Length: 792 Number of Matches: 1

Range 1: 28 to 60 [GenPept](#) [Graphics](#)

▼ Next Match ▲ Previous Match

| Score          | Expect | Method                   | Identities  | Positives   | Gaps     |
|----------------|--------|--------------------------|-------------|-------------|----------|
| 69.3 bits(168) | 3e-12  | Composition-based stats. | 33/33(100%) | 33/33(100%) | 0/33(0%) |

Query 1 IAPALIAVAPIAKYLATALAKWALKQGFAPLKS 33  
IAPALIAVAPIAKYLATALAKWALKQGFAPLKS  
Sbjct 28 IAPALIAVAPIAKYLATALAKWALKQGFAPLKS 60

# Tandem Repeats in a New Toxin Gene from *Bacillus thuringiensis* and in Other *cry11*-Like Genes

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## Key Words

Tandem repeats • *cry11Bb2* • *Bacillus thuringiensis*, a field isolate • Mosquito larvae

## Abstract

A new gene, *cry11Bb2* from a field isolate of *Bacillus thuringiensis*, was cloned for expression in *Escherichia coli*. The encoded protein, with a deduced molecular mass of 89.5 kDa, exhibits 97 and 79% identities with the overlap regions of Cry11Bb1 from *B. thuringiensis* ssp. *medellin* and Cry11Ba1 from ssp. *jegathesan*, respectively. It is however longer than Cry11Bb1 by 42 amino acids in its carboxy-terminus, of which 32 comprise 2 tandem repeats additional to the 5 existing in the latter polypeptide. Possible roles for this recurrent motif among Cry toxins and their accessory proteins, and for their encoding genes are proposed.

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Various subspecies of the aerobic, Gram-positive, endospore-forming bacterium *Bacillus thuringiensis* are better than all other microbial control agents against larvae of insect pests found to date. During sporulation, each ssp. produces large quantities of a certain combination of insect larvicidal proteins known as  $\delta$ -endotoxins that are aggregated in parasporal crystalline bodies [de

Maagd et al., 2003]. *B. thuringiensis*-based formulations have long been used intensively worldwide to control various insect pests [Van Frankenhuyzen, 1993].

Isolates of *B. thuringiensis* toxic to mosquito larvae have been classified into three groups by Delecluse et al. [2000]. Group 2 includes ssp. *medellin* and *jegathesan* that display crystal protein patterns different than that of ssp. *israelensis* and are nearly as toxic [Margalith and Ben-Dov, 2000], each contains a different Cry11 toxin: -Aa1 in *israelensis*, -Ba1 in *jegathesan*, and -Bb1 in *medellin*.

## Isolation and Characterization of a New *cry11Bb*-Like Open Reading Frame

Preliminary PCR screening of a series of *B. thuringiensis* field isolates [Ben-Dov et al., 1997] with a new pair of universal primers for *cry11*, Ucry11(d) (CCAGCAT-TAATAGCAGCTCC) and Ucry11(r) (GTACACATCT-GAGTAAAAAG), discovered several *cry11-like* genes. The pair Ucry11(d) and *cry11Bb*(r) (CAATATGCCAC-CAATATCT), designed for *cry11Bb1* from ssp. *medellin* [Ordaz et al., 1998], revealed a *cry11Bb*-like gene in isolate K34. When the pair of primers based on the sequence of

This article is dedicated to the memory of our colleague and friend, the late Professor Yoel Margalith.

**Fig. 1.** Tandem repeats of cry11Bb2. Nucleotides alignment of the 7 repeats, each encoding the 16 amino acids motif (top line; symmetrical tyrosines are bold-faced, and asparagines underlined). Mismatched nucleotides are marked in bold and italics. Codons of mismatched amino acids are underlined.

| N   | T   | S   | S   | G          | Y   | E   | Q   | G   | Y   | N          | D          | N   | Y   | N          | Q          |
|-----|-----|-----|-----|------------|-----|-----|-----|-----|-----|------------|------------|-----|-----|------------|------------|
| AAT | ACA | AGC | AGT | GGG        | TAT | GAG | CAA | GGA | TAT | AAC        | GAT        | AAT | TAT | AAC        | CAA        |
| AAT | ACA | AGT | AGT | GGG        | TAT | GAG | CAA | GGA | TAT | AAC        | GAT        | AAT | TAT | AAC        | CAA        |
| AAT | ACA | AGT | AGT | GGG        | TAT | GAG | CAA | GGA | TAT | AAC        | GAT        | AAT | TAT | AAC        | CAA        |
| AAT | ACA | AGT | AGT | GGG        | TAT | GAG | CAA | GGA | TAT | AAC        | GAT        | AAT | TAT | AAC        | CAA        |
| AAT | ACA | AGC | AGT | <u>GAG</u> | TAT | GAG | CAA | GGA | TAT | AAC        | <u>GAC</u> | AAT | TAT | AAC        | CAA        |
| AAT | ACA | AGT | AGT | <u>GGA</u> | TAT | GAG | CAA | GGA | TAT | AAC        | GAT        | AAT | TAT | AAC        | CAA        |
| AAT | ACA | AGT | AGT | <u>GGA</u> | TAC | GAG | CAA | GGA | TAT | <u>ATT</u> | GAT        | AAT | TAT | <u>AGG</u> | <u>CCA</u> |

*cry11Bb1*, 11B-Nco(d) (CAATAAATTTTAAGCAGGA-ACCATGGTAAATTC) and 11B-XhoBam(r) (GTATT-TTGGATCCTAAGTTCTCGAGTACCTTGCTCA-TACTCCACTGC), was used to amplify the whole gene, a truncated reading frame of 2,135 bp, with no stop codon, was discovered (accession # HM068615). This sequence (named  $\Delta$ *cry11Bb2*) was cloned for expression in *Escherichia coli* at the *NcoI*-*Bam*HI sites of pUHE-24S [Ben-Dov et al., 1995] to yield plasmid pHNt-B [Baranes-Sela, 2005]. The missing 3'-end of the gene was amplified with 11Bb1387(d) (CGCAGGGTCTTTACTA-TGGATGGAATA; starting from nucleotide # 1,387) and 11Bb-Bam(r) (AAAACGGATCCATAATGTGCTTGG) downstream *cry11Bb1* [Orduz et al., 1998]. The 1,530-bp amplicon containing the native *KpnI* site (nt 1,448–1,453) was ligated into pHNt-B by replacing its shorter (687 bp) *KpnI*-*Bam*HI fragment to yield pHN-B with 2,874 bp encompassing the whole new *cry11Bb1*-like ORF (between nucleotides 210–2,588).

The new ORF (2,379 bp; accession # HM068615) was analyzed (on both strands) with DNA sequencer ABI model 373A System (Applied Biosystems). Two putative ribosome-binding sites with adjacent in-frame ATG codons were found in *cry11Bb1* [Orduz et al., 1998] but in vivo translation in *B. thuringiensis* ssp. *medellin* starts at the second (accession # AF017416). The first ORF is indeed excluded in the new *cry11Bb2* because it contains two tandem T's (# 141–142; accession # HM068615) rather than 3 in *cry11Bb1* that would form a short ORF (9 amino acids long) from the first ATG, concordant with Cry11Ba1 and Cry11Aa1. This may stem from an artifact "T" in *cry11Bb1*. The identity of the N-terminal amino acids of both Cry11Bb's (-1 and -2) confirms this explanation.

The N-terminus of the deduced encoded protein of 792 amino acids with a molecular mass of 89.486 kDa and pI of 5.5 (<http://workbench.sdsc.edu>) is 60% identical to Cry11Aa1 of *B. thuringiensis* ssp. *israelensis* (overlapping 651 amino acids), 79% to Cry11Ba1 of ssp. *jegathesan*

(overlapping 728 amino acids) and 97% to Cry11Bb1 of ssp. *medellin* (overlap of 750 amino acids). The protein was named Cry11Bb2 (accession # HM068615), but the conventional nomenclature [Crickmore et al., 1998] does not take into account additional amino acids at protein termini.

Western blot analysis (using antibodies against Cry11Aa1, kindly provided by Sarjeet Gill) of the recombinant *E. coli* strains expressing full-length *cry11Bb2* and its truncated version from pHN-B and pHNt-B respectively, displayed about 110 and 70 kDa proteins (data not shown). Cross-reaction of Cry11Ba1 has previously been observed with anti-Cry11Bb1 [Delecluse et al., 1995; Orduz et al., 1998] and even with antibodies raised against the more distant Cry4Aa1 [Delecluse et al., 1995]. Toxicities to early 3rd instar *Aedes aegypti* larvae of the same recombinant *E. coli* strain [as in Ben-Dov et al., 1995] expressing the truncated or the intact *cry11Bb2* were comparable to that of *cry11Aa1* [Baranes-Sela, 2005; Khasdan et al., 2001; data not shown].

### Repeated Motifs

The 126 bp at the 3'-end of *cry11Bb2*, additional to *cry11Bb1*, completing a sequence of 7 tandem repeats of 16 amino acids (NTSSGYEQGYNDNYNQ) near the C-terminus (fig. 1), are located between amino acids 665–776 of the deduced sequence (accession # HM068615). The 5th repeat contains a single mismatch (at amino acid # 733) and the last has 3 (# 771, 775 and 776). Cry11Bb1 (accession # AF017416) includes 5 such regions, whereas Cry11Aa1 (accession # AL731825) contains none, and Cry11Ba1 (accession # X86902) has 3 with varying numbers of mismatches.

Repeated blocks have been found in other mosquito larvicidal  $\delta$ -endotoxins [de Maagd et al., 2003]: Cry20Aa and Cry27Aa. The roles of such tandem repeat motifs are however unknown.

**Table 1.** Correlation between number of repeats and observed molecular mass in currently known versions of Cry11

| Cry11 version  | Derived MW, kDa | Observed MW, kDa | $\Delta$ MW (= obs – der) (% difference) | Repeats, n | Reference              |
|----------------|-----------------|------------------|------------------------------------------|------------|------------------------|
| –Bb2           | 89.5            | 110              | 20.5 (22.9%)                             | 7          | This study             |
| –Bb1           | 88.2            | 94               | 5.8 (6.6%)                               | 5          | Orduz et al., 1998     |
| –Ba1           | 81.3            | 80               | –1.3 (0.0%)                              | 3          | Delecluse et al., 1995 |
| –Aa1           | 72.3            | 65               | –7.3 (–10.1%)                            | 0          | Dai et al., 1993       |
|                |                 | 72               | 0 (0%)                                   | 0          | Donovan et al., 1988   |
| – $\Delta$ Bb2 | 77.2            | 70               | –7.2 (–9.3%)                             | 0          | This study             |

MW = Molecular weight.

The highly conserved 16-mer motif of 7 tandem repeats in Cry11Bb2 (accession # HM068615) is reflected in a similar conservation of its encoding nucleotide sequence of 48-mer motif (fig. 1). In particular, only 6 transitions exist among the 288 nucleotides composing the first 6 repeats, one of which alone encodes a different amino acid (G→E). The other 6 modifications, all in the last (7th) repeat, contain 3 transversions. Intriguingly, the degree of accuracy in repeat sequence is lower in the last copy in both amino acids and nucleotides. The latter may result of an in vivo mechanism that mimics the in vitro microgene polymerization reaction [Itsko et al., 2009, 2011].

The SDS-PAGE-observed molecular mass (110 kDa; not shown) of the full-length Cry11Bb2 is higher by ca. 20% than the deduced (89.5 kDa; accession # HM068615). This difference is significantly larger than the analogous differences observed with other Cry11 versions (table 1). The ORF for the truncated polypeptide  $\Delta$ Cry11Bb2 predicts a protein of 77,196 kDa – about 9% higher than the sequence-derived mass. The mobility of currently known Cry11 proteins is inversely related to the copy number of the repetitive motif (table 1). Orf2 from *B. thuringiensis* ssp. *kurstaki* displays an even larger anomaly: its electrophoretic mobility corresponds to a molecular mass of 50 kDa despite a predicted size of 29 kDa [Widner and Whiteley, 1989]. This phenomenon was also recorded in small spore-coat proteins of *Bacillus subtilis* [Zhang et al., 1993]. A higher apparent molecular weight may be due to residual secondary structures of these proteins retarding migration on the gel [Manasherob et al., 2001]. The question whether it means that the repeated motif (NTSSGYEQGYNDNYNQNTSSGYEQGYNDNYNQ) imposes some constraint on the denatured protein remains moot, but its symmetries around 3 tyrosine residues

(Y\*\*\*Y\*\*\*Y) and 4 asparagines (N\*N\*N\*N) are noteworthy. The possible roles of repeated motif in protein structure, stability and mobility are still to be resolved.

Repeating motifs in natural proteins are widely documented and described (<http://www.ncl-india.org/trips/>), and their possible activity mechanisms discussed [Katti et al., 2000]. The fact that they derive of encoding genes is sometimes not sufficiently considered. During studies with the entomopathogenic *B. thuringiensis* subspecies, we encountered several such cases where DNA repeats exist, reflecting their encoded protein motifs. Specifically, helper Orf2 proteins of some *cry* operons, *cry2Aa1* [Widner and Whiteley, 1989], *cry2Ac1* [Wu et al., 1991] and *cry39Aa1* [Ito et al., 2002] include different numbers of repetitive motifs. For example, Orf2<sub>cry2Aa</sub>, encoded by *orf2* of the *cry2Aa* operon, contains 11 tandem repeats of a 15-amino-acid motif and accounts for two-thirds of all its residues [Widner and Whiteley, 1989]. The suggestion that Orf2 is involved in Cry2 crystallization [Crickmore and Ellar, 1992] was later confirmed for Cry2Aa1 [Ge et al., 1998; Staples et al., 2001]. This motif is encoded by a 45-nt repeat in its gene *orf2*<sub>cry2Aa</sub> (fig. 2a) that includes only 6 transitions and a single transversion among the 450 nt composing the first 10 repeats. All 5 additional changes are located at the 3' end of the last (11th) repeat. *orf2*<sub>cry2Aa3</sub> [Sasaki et al., 1997] (fig. 2b) includes 13 repeats with the same basic unit as *orf2*<sub>cry2Aa</sub>. In this gene, the first 9.5 repeats are separated from the rest by a 12-nt long 'linker' (highlighted dark gray with black letters in figure 2b).

A second type of repeat unit among the known *orf2* genes is found in *orf2*<sub>cry2Ca</sub> [Wu et al., 1991] and in *orf2*<sub>cry2Ac</sub> (accession # AY007687). It differs from the repeat of *orf2*<sub>cry2Aa</sub> by two triplets that surround a conserved T(A/G)C found in both kinds of repeats and by an

[illegible]

ACG TAT AAC CAA AGT CAG AAT GTA TGC GCG CAA GAT TTA GTT GAT  
 ACG TAT AAC CAA AGT CAG AAT GTA TGC ACA CAA GAT TTA GTT GAT  
 ACG TAT AAC CAA AGT CAG AAT GTA TGC ACA CAA GAT TTA GTT GAT  
 ACG TAT AAC CAA AGT CAG AAT GTA TGC ACA CAA GAT TTA GTT GAT  
 ACG TAT AAC CAA AGT CAG AAT GTA TGC ACA CAA GAT TTA GTT GAT  
 ACG TAT AAC CAA AGT CAG AAT GTA TGC GCG CAA GAT TTA GTT GAT  
 ACG TAT AAC CAA AGT CAG AAT GTA TGC ACA CAA GAT TTA GAA GAT  
 ACG TAT AAC CAA AGT CAG AAT GTA TGC ACA CAA GAT TTA GTT GAT  
 ACG TAT AAC CAA AGT CAG AAT GTA TGC GCG CAA GAT TTA GTT GAT  
 ACG TAT AAC CAA AGT CAG AAT GTGGTCTTACACAG CAA GAT TTA GTT GAT  
 ACG TAT AAC CAA AGT CAG AAT GTA TGC GCG CAA GAT TTA GTT GAT  
 ACG TAT AAC CAA AGT CAG AAT GTA TGC ACA CAA GAT TTA GTT GAT  
 ACG TAT AAC CAA AGT CAG AAT ACT TAT ATA CAA GAT TTA ATT GAT  
**b** AAG TAT AAT CAA AGT CAG AAT

|   |     |     |     |     |     |     |     |       |     |     |     |     |     |     |     |     |
|---|-----|-----|-----|-----|-----|-----|-----|-------|-----|-----|-----|-----|-----|-----|-----|-----|
| C | ACG | TAT | AAC | CAA | AGC | CAA | AAT | GGCTG | TAC | GCG | CAA | GAT | TTA | GTG | GAT |     |
|   | ACG | TAT | AAC | CAG | AGC | CAA | AAT | GGT   | GCC | TGC | GCG | CAA | GAT | TTA | ATG | GAT |
|   | ACG | TAT | AAC | CAA | AGC | CRA | AAT | GGT   | GCC | TAC | GCG | CAA | GAT | TTA | GTG | AAT |
|   | ACG | TAT | AAC | CAA | AGT | CAG | AAT | GGT   | GCC | TAC | GCG | CAA | GAT | TTA | GTG | GAT |
|   | ACG | TAT | AAT | CAA | AGT | CAG | AAT | GGT   | GCC | TGC | GCG | CAA | GAT | TTA | ATG | GAT |
|   | ACG | TAT | AAC | CAA | AGC | CRA | AAT | GGT   | GCC | TAC | GCG | CAA | GAT | TTA | GTG | AAT |
|   | ACG | TAT | AAC | CAA | AGT | CAG | AAT | GGT   | GCC | TAC | GCG | CAA | GAT | TTA | GTG | GAT |
|   | ACG | TAT | AAT | CAA | AGT | CAG | AAT | GGT   | GCC | TGC | GCG | CAA | GAT | TTA | ATG | GAT |
|   | ACG | TAT | AAC | CAA | AGC | CRA | AAT | GGT   | GCC | TAC | GCG | CAA | GAT | TTA | GTG | AAT |
|   | ACG | TAT | AAC | CAA | AGT | CAG | AAT | GGT   | GCC | TAC | GCG | CAA | GAT | TTA | GTG | GAT |
|   | ACG | TAT | AAT | CAA | AGT | CAG | AAT | GGT   | GCC | TGC | GCG | CAA | GAT | TTA | GTG | GAT |
|   | ACG | TAT | AAC | CAA | AGC | CRA | AAT | GGT   | GCC | TAC | GCG | CAA | GAT | TTA | GTG | AAT |
|   | ACG | TAT | AAC | CAA | AGT | CAG | AAT | GGT   | GCC | TAC | GCG | CAA | GAT | TTA | GTG | GAT |
|   | ACG | TAT | AAT | CAA | AGT | CAG | AAT | GGT   | GCC | TGC | GCG | CAA | GAT | TTA | GTG | GAT |
|   | ACG | TAT | AAC | CAA | AGT | CAG | AAT | GGT   | GCC | TAC | GCG | CAA | GAT | TTA | GTG | AAT |
|   | ACG | TAT | AAC | CAA | AGT | CAG | AAT | GGT   | GCC | TAC | GCG | CAA | GAT | TTA | GTG | GAT |
|   | ACG | TAT | AAT | CAA | AGT | CAG | AAT | GGT   | GCC | TGC | GCG | CAA | GAT | TTA | GTG | GAT |
|   | ACG | TAT | AAC | CAA | AGT | CAG | AAT | GGT   | GCC | TAC | GCG | CAA | GAT | TTA | GTG | AAT |
|   | ACG | TAT | AAC | CAA | AGT | CAG | AAT | GGT   | GCC | TAC | GCG | CAA | GAT | TTA | GTG | GAT |
|   | ACG | TAT | AAT | CAA | AGT | CAG | AAT | GGT   | GCC | TGC | GCG | CAA | GAT | TTA | GTG | AAT |
|   | ACG | TAT | AAT | CAA | AGT | CAG | AAT | GGT   | GCC | TGC | GCG | CAA | GAT | TTA | GTG | GAT |
|   | ACG | TAT | AAC | CAA | AGT | CAG | AAT | GGT   | GCC | TAC | G   |     |     |     |     |     |

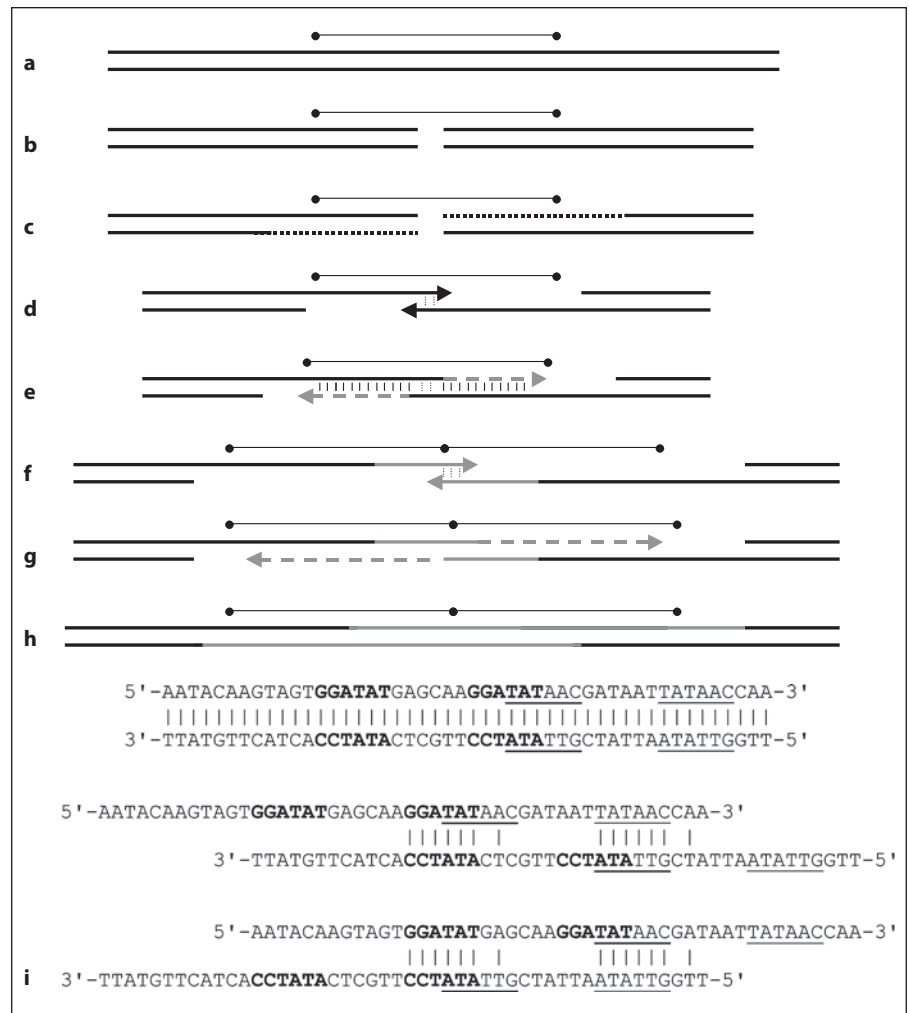
**d**

|     |     |      |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| ACG | AAT | AAC  | CAA | AGT | CAG | AAT | GGT | GCC | TAC | GCG | CAA | GAT | TTA | GTG | GAT |
| ACG | TAT | TAAC | CAA | AGT | CAG | AAT | GGT | GCC | TAC | GCG | CAA | GAT | TTA | AGT | GAT |
| ACG | TAT | AAT  | CAA | AGT | CAG | AAT | GGG | CCC | TAC | GCG | CAA | AAT | TTA | GTG | GAT |
| ACG | TAT | AAT  | CAA | AGT | CAG | AAT | GGT | CCC | TAC | GCG | CAA | GAT | TTA | GTG | AAT |
| ACG | TAT | AAT  | CAA | AGT | CAG | AAT | GGT | GCC | TAC | GCG | CAA | GAT | TTA | GTG | GAT |

[illegible]

**Fig. 2.** Individually aligned repeated motifs found in different *B. thuringiensis* (Bt) subspecies. **a** *orf2*<sub>cry2Aa</sub> from Bt ssp. *kurstaki* HD-1. **b** *orf2*<sub>cry2Aa3</sub> from Bt ssp. *sotto*. **c** *orf2*<sub>cry2Ca</sub> from Bt S1. **d** *orf2*<sub>cry2Ac</sub> from Bt ssp. *thuringiensis*. **e** *cry11Bb2* from Bt strain K34. Fonts: black, bold, italics – pyrimidine transitions; black, bold – purine transitions; black, bold, underlined – transversions. Highlights: light gray with white letters, the conserved triplet in the middle of the motif; light gray with black letters, region of varied length (modulus 3); black with white letters, sequence homology between *cry11Bb2* and *orf2*; dark gray with black letters, non-homologous insertions; N = non-identified nucleotides.

**Fig. 3.** Generation of sequence duplication due to DSB repair via the NHEJ-like pathway. **a, b** Chromosome region (**a**) suffers a DSB (**b**). **c** Trans-resection of DNA strands flanking the break by a dedicated exonuclease. **d** Bridging the broken ends in staggered mode by occasional Watson-Crick bonds due to partial complementarity. **e** Filling-in single-stranded regions by DNA polymerase. **f** Back slippage of the broken ends under repair that is mediated by a weak 2nd rank complementarity. **g** Filling-in single-stranded regions by DNA polymerase. **h** Restored integrity of the chromosome with the generated duplicated region. Dumbbells designate schematic location of the sequence undergoing duplication. Newly synthesized DNA is designated in gray. **i** Fully aligned and two slipped structures existing between constituent strands of *cry2Bb2* repeat motif that may assist to bridge the DSB ends. Bold and underlined regions designate two sets of complementarity.



extra adjacent triplet. A string of 991 nt in *orf2<sub>cry2Ca</sub>* encoding almost 21 repeats of a 16-mer motif in *Cry2Ca* contains only 35 transitions (fig. 2c), whereas the shorter (7 repeats) string in *orf2<sub>cry2Ac</sub>* is much less conserved and contains many deletions and insertions (fig. 2d).

Addition of four endogenous nucleotides to the 5' end *cry11Bb2* encoding nucleotide sequence as displayed in figure 1, arranges 7 tandem repeats of 48-mer motif somewhat differently (fig. 2e). Seven (highlighted black with white letters) out of 16 triplets constituting the repetitive motif of *Cry11Bb2* are identical/homologous in composition and location to those found in previously described *orf2* genes (fig. 2a-d).

The spectrum of replacements in the repeats of *orf2<sub>cry2Aa</sub>* and *orf2<sub>cry2Ca</sub>* is reciprocal on two sides of the conserved T(A/G)C triplet: in its 5' part there is a bias to pyrimidine transitions whereas purine transitions are ex-

clusively observed at its 3' part. Intriguingly, such pattern of changes may point to occurrence of double strand break (DSB) in the middle of the repeat. Repair of DSB is mediated by trans-resection of opposite strands surrounding it in a way that transiently exposes generated ssDNA stretches to increased mutagenesis with characteristic pattern of transitions encompassing the DSB [Yang, 2008]. Moreover, considerable evidence has accumulated in eukaryotic cells that point to blunt dsDNA ends as key intermediates in the process leading to gene amplification [Mondello et al., 2010; Pace et al., 2009].

One of the error-prone processes exploited by cells to survive DSB is non-homologous end-joining (NHEJ) [Mahaney et al., 2009] that may contribute to sequence amplifications. It was regarded as exclusive prerogative of eukaryotic cells. However, it has recently been found in prokaryotes by identifying bacterial homologues of Ku

protein [Brissett and Doherty, 2009; Doherty et al., 2001]. Moreover, a functional NHEJ repair pathway is essential for spore viability in *B. subtilis* under conditions that yield DSBs [Weller et al., 2002; Moeller et al., 2007]. It is noteworthy that many of the bacteria that contain the Ku ligase system are capable of sporulation (*B. subtilis*, *Streptomyces coelicolor*) or spend long periods of their life cycle in the stationary phase (*Mycobacterium tuberculosis*, *Mesorhizobium loti*, *Sinorhizobium loti*) [Weller et al., 2002]. The sporulating *B. thuringiensis* may contain a NHEJ repair pathway as well.

Exogenous oligonucleotides complementary to the broken ends can efficiently target DSB for repair in yeasts [Storici et al., 2006]. We have recently proposed [Itsko et al., 2011] that partial complementarity between sequences flanking the broken ends may assist their sealing and contribute to repeat generation via slipped structures (fig. 3a–h). An example involving putative 2nd rank complementarity that can participate in the process to seal the broken ends was revealed in Cry2Bb2 (fig. 3i).

Once the initial doublet has been generated inside the chromosome, the repeats may propagate during DNA replication since the constituent strands may slide over each other between the multiple complementary regions. The alternative mechanism for the propagation is possible recurrence of DSBs in the middle of the generated repeats. Bridging the broken ends by occasional Watson-Crick bonds due to partial complementarity (fig. 3d) can bring about nucleotides insertions/deletions during fill-in by DNA polymerase. Selection for functionality of a given coding region can restore the lost frame by generating an additional triplet or dropping an existing one. This can result in the two/three triplets varied region (highlighted light gray with black letters in figure 2) surrounding the conserved T(A/G)C triplet in the 4 *orf2* versions and in *cry11Bb2* listed here (highlighted light gray with white letters in figure 2). The inserted linker in that region as in *orf2<sub>cry2Aa3</sub>* (highlighted dark gray in figure 2b) is also consistent with this explanation.

It is intriguing that the AGT triplet in *orf2<sub>cry2Ca</sub>* (fig. 2c) is always adjacent to CAG whereas its 5'-replaced AGC is adjacent to CAA. Both combinations encode the same amino acids: serine by AGT/C and glutamine by CAG/A. This coincidence cannot be explained by selection for suppression of one transition by the other. Such replacements do affect stability of slipped structures presumably emerging during replication of this region. Without the replacements, almost triplet repetition AAT CAA AGT CAA AAT (fig. 2c) would be explicitly observed that could putatively propagate in an uncontrolled way

through slipped structures, bringing about genetic instability. Strong selection for silent transitions that save triplet coding but prevent deleterious expansion by disrupting repetition pattern would thus be anticipated to stabilize the genome.

A sequence of 13 bp long (bp 2,601–2,613; accession # HM068615) starting 12 bases downstream the stop codon shares 100% identity with those of the 16-bp inverted repeat of the insertion sequence IS240A that has initially been found to flank *cry4Aa1* [Delecluse et al., 1989]. Most *B. thuringiensis* ssp. tested, including all known mosquito-cidal strains, possess at least one IS240-related element [Rosso and Delecluse, 1997]. IS240 represents a family of insertion sequences with several variants [Leonard et al., 1997] that might be involved in the dispersion of toxin genes among various *B. thuringiensis* strains [Leonard et al., 1997; Rosso and Delecluse, 1997]. They may also be instrumental to duplicate such motifs as found here.

## Concluding Remarks

A new representative of Cry proteins that contains tandem repeats, Cry11Bb2, was discovered, described and discussed. The high degree of conservation of nucleotide repeats in *cry11Bb2* that code for the corresponding amino acids tandem repeats, points to their possible generation from a common template apparently by slippage between partly complementary regions during DNA replication. The motif repetitions generate periodic structures. Proteins that contain such a structure can serve as scaffolding centers, which assist toxin crystallization during the sporulation stage.

Molecular participants in the repeat expansion process and reaction conditions accompanying it in vivo may shed further light on the basic genetic mechanism as well as the crystallization process, stability and toxicity of the  $\delta$ -endotoxins.

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## BTM-P1 polycationic peptide biological activity and 3D-dimensional structure

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### Abstract

The novel BTM-P1 peptide interferes with energetic processes in mitochondria; its antimicrobial activity against Gram-positive and Gram-negative bacteria is described here. BTM-P1 three-dimensional structure was determined by <sup>1</sup>H NMR to explain its biological mechanisms and membrane activity. Structural data indicated that BTM-P1 can form an  $\alpha$ -helix; circular dichroism analysis confirmed the peptide's propensity to behave as a typical transmembrane helix in a lipidic environment. According to the structural characteristics of the polycationic BTM-P1 peptide so revealed, its biological activity can be explained by a mechanism involving the formation of ion-permeable channels in biomembranes.

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Cationic antimicrobial peptides are generally peptides having less than 50 amino acid residues with an overall positive charge imparted by the presence of multiple lysine and arginine residues and a substantial portion (50% or more) of hydrophobic residues [1]. These peptides are produced by all kinds of organism (from plants and insects to humans) [2] as a major part of their innate effective non-specific defences against infection [3]. They are preferentially amphipathic allowing the peptide to interact with plasma membrane phospholipids [4]. Several of these molecules have been isolated, amino acid sequenced and described in the literature. Along with their antimicrobial activity, these peptides have been shown to inhibit certain viruses [5], have anticancer activity [6], promote wound healing

[7] and cause depolarisation of isolated mitochondria's inner membrane [8]. These properties make cationic peptides very promising candidates for developing new therapeutic agents.

It is generally assumed that most antimicrobial peptides disrupt or permeate the target cell membrane, resulting in irreversible damage of membrane functionality [9]. Most cationic peptides seem to adopt an amphipathic arrangement with opposing hydrophobic and positively charged faces when coming into contact with the bacterial membrane [10]. Accumulating data suggests that the permeating pathway depends on both the peptide and the membrane. More specifically, membrane-bound peptides recognise each other, oligomerise and form transmembranal pores. Permeation/disruption mechanism can vary amongst different peptides.

The Cry toxins' pore-forming domain consists of seven  $\alpha$  helices presenting lytic peptide features [11]. One of

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the peptides (BTM-P1), synthesised based on Cry11Bb1 *Bacillus thuringiensis* serovar. *Medellin* toxin  $\alpha 2$  helix, has been described [12] and its activity on isolated mitochondria has been studied recently [13]. BTM-P1's ability to kill bacteria was evaluated, the 3D-peptide structure was solved, its conformation in solution and in hydrophobic environment determined and a mechanism for its biological activity proposed.

## Materials and methods

**Chemical synthesis of peptides.** All the peptides were synthesised by the solid-phase method [14] according to good manufacturing practices (GMP). A *p*-methyl benzhydrylamine-resin (MBHA) (0.7 Meq/g), *t*-Boc aminoacids (Bachem, USA) and low-high cleavages were used in the process [15]. Once synthesised, the peptides were extracted with 10% (v/v), acetic acid and water. The peptides were purified by reverse-phase HPLC, lyophilised, and dissolved in bidistilled water at 1 mg/mL concentration.

**Mitochondria isolation.** White male rats were decapitated and their livers homogenised in 45 mL ice-cold medium composed of 210 mM mannitol, 70 mM sucrose, 5 mM Hepes–Tris, and 1 mM EGTA, pH 7.4, using a glass Dounce homogeniser and Teflon pestle. The homogenate was centrifuged at 600g for 10 min at 4 °C (Jouan centrifuge, type MR1812). The supernatant was again centrifuged at 10,000g for 10 min at 4 °C. The mitochondrial pellet was suspended in 30 mL ice-cold 210 mM mannitol, 70 mM sucrose, 5 mM Hepes–Tris, and 20  $\mu$ M EGTA, pH 7.4 (MSHE) and further centrifuged at 10,000g for 10 min at 4 °C. The final mitochondrial pellet was suspended in MSHE buffer at 60–80 mg/mL protein concentration. All analytical grade reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

**Mitochondrial swelling assays.** Mitochondrial swelling was monitored as changes in apparent absorbance at 540 nm ( $OD_{540}$ ) on a Turner spectrophotometer (model SP-850, USA) modified with a mini-magnetic stirrer device and a thermostated chamber. The curves were recorded on a graphic register (Linseis model L250E, USA). 0.5 mg/mL mitochondria, 2.5  $\mu$ M rotenone and 1  $\mu$ M oligomycin (RO) were simultaneously added to 1 mL of different isotonic media: 125 mM  $NH_4NO_3$ , 5 mM Hepes–Tris, pH 7.4; 125 mM Tris- $NO_3$ , 5 mM Hepes–Tris, pH 7.4; 125 mM  $NaNO_3$ , 5 mM Hepes–Tris, pH 7.4; 125 mM  $KNO_3$ , and 5 mM Hepes–Tris, pH 7.4.

**Antimicrobial activity assays.** The peptide's antimicrobial activity was tested against different bacteria (see Table 2). Bacterial growth proceeded until logarithmic phase (4–6 h) at 1.0  $OD_{600}$ . Spent culture medium was removed by spinning at 10,000 rpm for 10 min at 4 °C (International Equipment Company IEC, Centra MP4R; rotor 851, USA); the bacterial pellet was washed twice in 25 mM  $Na_2PO_4$ , 25 mM  $NaHPO_4$ , 150 mM NaCl, and 5 mM KCl, pH 7.4 (PBS). Bacterial concentration was adjusted to a 0.2  $OD_{600}$  in the same buffer (5000–10,000 cells/mL). The peptide's *in vitro* antibacterial activity was measured as follows: 500  $\mu$ L bacterial suspension were treated with 7.1  $\mu$ M of BTM-P1 peptide for 2 h at 37 °C in 1.5 mL Eppendorf tubes. Following incubation, cells were washed in the same volume of buffer and then plated onto nutrient agar Petri dishes; colony forming units (CFU) were determined after 24 h incubation at 37 °C. Percentage inhibition was calculated by comparing the number of CFU obtained when incubated with and without the peptide.

**Circular dichroism (CD).** Spectra were performed as described. [16] Measurements were taken at room temperature on nitrogen-flushed cells using a Jasco J-810 spectropolarimeter (Jasco, Japan). Spectra were recorded at 190–250 nm wavelength interval using a 1-mm path-length rectangular cell. Each spectrum was obtained by averaging three scans taken at a 20 nm/min scan-rate with 1 nm spectral bandwidth; Jasco software was used for correcting the baseline. The CD profiles were done by dissolving lyophilised purified peptides in 0–50% aqueous 2,2,2-trifluoroethanol (TFE) in a final 500  $\mu$ L volume. A typical 0.2  $\mu$ M peptide concentration in TFE-water mixture stabilised but did not induce secondary structure in peptides, as described elsewhere [17]. The results were expressed as molar ellipticity.

**Liposome preparation.** Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and cholesterol (CH) were dissolved in chloroform at 1:1:1 PC:PE:CH and 1:1 PC:CH molar ratios. Solvent was then removed by evaporation and dried at high vacuum for 8 h. The lipid mixture was rehydrated to final 100 mM concentration in 25 mM  $Na_2PO_4$ , 25 mM  $NaHPO_4$ , 150 mM NaCl, and 5 mM KCl, pH 7.4 (PBS). Liposomes were homogenised by sonication (Sonicator Branson, USA) for 1 h. Fifty millimolar phospholipids (liposomes) and 0.2  $\mu$ M peptide in PBS were mixed for 1 h at 5 °C and analysed by CD at room temperature, as described above.

**NMR analysis.** NMR samples were prepared by dissolving 7–10 mg of peptide in 500  $\mu$ L TFE- $d_3$  (Cambridge Isotope, 99.94%)/ $H_2O$  mixture (30/70 v/v). NMR spectra were recorded on a Bruker DRX-600 spectrometer (Bruker, Billerica, USA) at 295 K. Basic NMR structure determination protocol for all peptides was as follows: proton spectra were assigned by DQF-COSY [18], TOCSY [19] and NOESY [20]; TOCSY and DQF-COSY spectra were then used for identifying individual spin systems (amino-acids) and NOESY (350 ms mixing time) for amino-acid stretches within a given primary structure (sequential assignment). Three-dimensional structure was assigned by Wüthrich's method [21]. 2D NMR data were processed with XWIN-NMR software. TOCSY spectra, recorded at different temperatures (285–315 K), were used to obtain amide temperature coefficients for predicting hydrogen bonds ( $-\Delta\delta H^N$ /ppb/K).

**Structure calculations.** Peptide structure was determined by Accelrys Inc., software. NOESY peak signals were classified as strong, medium and weak according to their relative intensity corresponding to 1.8–2.5, 2.5–3.5 and 3.5–5.0 Å interproton distances, respectively. Hydrogen bond constraints were introduced for low amide temperature coefficients; only  $<4$  and  $-\Delta\delta H^N$ /ppb/K were used in structure calculations. Distance ranges involving these likely  $NH\cdots O$  hydrogen bonds were set at 1.8–2.5 Å, between residue acceptor oxygen (i-4) and residue donor amide hydrogen (i). All peptide bonds were forced to trans and  $C\alpha$  chirality to L during calculations. Distance Geometry (DGII) software was used for providing a family of 50 structures. These structures were refined by using simulated annealing protocol (Discover software). Structures having reasonable geometry and few violations were then selected.

## Results

### Effect of Cry11Bb1-derived peptides on mitochondrial swelling

Only the BTM-P1 peptide displayed significant ionophore activity in the inner mitochondrial membrane amongst the peptides tested here (Table 1, Fig. 1A and B). BTM-P1's ionophoric activity was determined in mitochondria suspended in isotonic 125 mM  $KNO_3$ , 5 mM Hepes–Tris pH 7.4 (Fig. 1A) and 125 mM  $NH_4NO_3$ , 5 mM Hepes–Tris pH 7.4 (Fig. 1B) media at 1.8, 0.36 and 0.18  $\mu$ M concentration of BTM-P1 (Fig. 1A and B, traces a–c, respectively). The rate of swelling (slopes) induced by BTM-P1 in 125 mM  $KNO_3$ , 5 mM Hepes–Tris, pH 7.4 was comparable to that of valinomycin (–1.6 slope with 0.18  $\mu$ M BTM-P1 and –1.4 slope with 120 nM Valinomycin) (Fig. 1A, traces c and e, respectively). A significant increase in mitochondrial swelling rate was observed when mitochondria was energised (–9.3 slope with 0.18  $\mu$ M BTM-P1 and 2.5 mM succinate) (Fig. 1A, trace d). BTM-P1 swelling rate in 125 mM  $NH_4NO_3$ , 5 mM Hepes–Tris, pH 7.4, was comparable to that of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazine (FCCP) (–2.1 slope with 0.36  $\mu$ M BTM-P1 and –3.5 slope with 2  $\mu$ M FCCP) (Fig. 1B traces b and f, respectively). Swelling

Table 1  
Amino acid sequence for the synthetic peptides corresponding to some of the domain I regions of the Cry11Bb1 toxin

| Peptide No. | Sequence                                                       | Helix* | Molecular weight |
|-------------|----------------------------------------------------------------|--------|------------------|
| BTM-P1      | <sup>35</sup> VAPIAKYLATALAKWALKQGFAKLKS <sup>60</sup>         | α1     | 2788             |
| BTM-P8      | <sup>24</sup> IEPSIAPALIAVAPIAKYLATAL <sup>46</sup>            | Δα2a   | 2307             |
| BTM-P9      | <sup>47</sup> AKWALKQGFAKLKS <sup>60</sup>                     | Δα2α   | 1576             |
| BTM-P10     | <sup>178</sup> PEDKDSLICQFNRYVNEYNTRMMGLYSIEFGR <sup>209</sup> | α6     | 3890             |
| BTM-P11     | <sup>79</sup> QTILNQTLQTDRVATLKAIEYEGFIHLGKV <sup>107</sup>    | α3     | 3287             |

\* α-Helices from Gutierrez et al. [22].

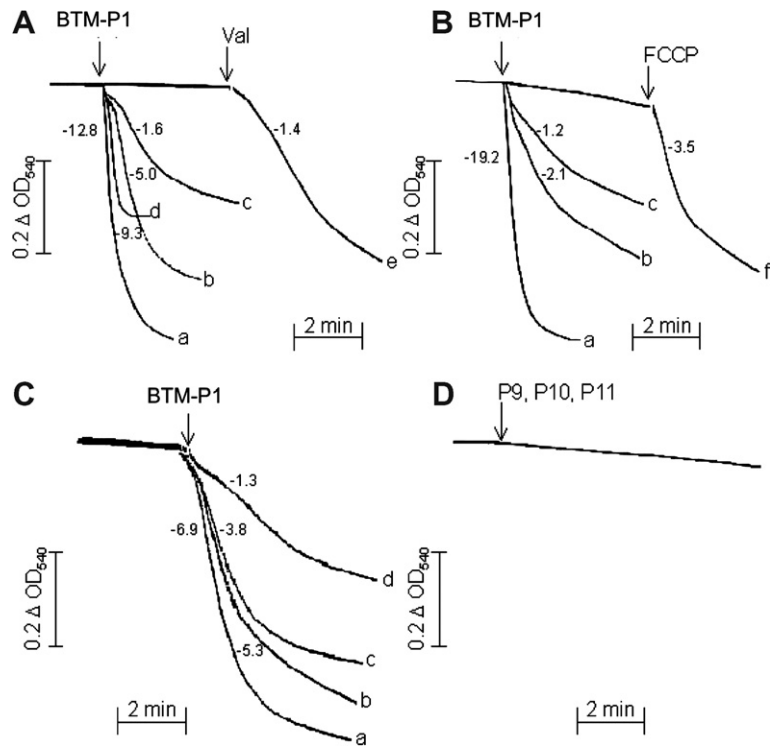


Fig. 1. Peptide BTM-P1 ionophore activity on the inner mitochondrial membrane. Mitochondria at 0.5 mg/mL protein concentration were incubated in isotonic KNO<sub>3</sub> (A), NH<sub>4</sub>NO<sub>3</sub> (B), both media were supplemented with Rotenone and Oligomycin (RO). BTM-P1 peptide was added at different concentrations: (trace a) 1.8 μM BTM-P1; (trace b) 0.36 μM BTM-P1; (trace c) 0.18 μM BTM-P1 and (trace d) 2.5 mM succinate and 0.18 μM BTM-P1, (trace e) 120 nM valinomycin; and (trace f) 2 μM FCCP. BTM-P1 ion selectivity (C): 0.18 μM BTM-P1 was added to mitochondria plus RO in isotonic NH<sub>4</sub>NO<sub>3</sub>, KNO<sub>3</sub>, NaNO<sub>3</sub>, Tris-HCl, (traces a–d, respectively). The effect of other peptides on mitochondrial swelling was determined in isotonic NH<sub>4</sub>NO<sub>3</sub> (D); P9, P10 and P11 peptide activity is shown.

rate in the presence of BTM-P1 depended on the media in the following order: NH<sub>4</sub>NO<sub>3</sub> > KNO<sub>3</sub> > NaNO<sub>3</sub> > Tris-NO<sub>3</sub> (Fig. 1C slopes in traces a–d, respectively). The results suggest that BTM-P1 may form ion channels in the inner mitochondrial membrane thus uncoupling the oxidative phosphorylation system. Curves obtained with P9, P10, and P11 peptides are shown in Fig. 1D. The remaining peptides including truncated versions of BTM-P1 (P8 and P9 in Table 1) did not show ionophore activity.

Antimicrobial activity

*Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pneumoniae* growth was completely inhibited (100%)

with 7.1 μM BTM-P1 peptide. *Haemophilus influenzae*, *Pseudomonas aeruginosa* and a *P. aeruginosa* multiresistant strain to antibiotics were susceptible to the same concentration of the peptide (7.1 μM BTM-P1) displaying 76% and 86% growth inhibition. *Klebsiella pneumoniae* was less susceptible at 7.1 μM peptide concentration, displaying 30.8% and 40% growth inhibition. Table 2 shows BTM-P1's antimicrobial spectrum, demonstrating that the BTM-P1 has a definite killing effect on bacteria.

BTM-P1 conformation (CD)

BTM-P1 peptide solution structure, as determined by spectroscopic circular dichroism (CD), indicated that it

Table 2  
BTM-P1 peptide antimicrobial activity

| Bacterium                       | Strain            | Dose<br>μM | % growth<br>inhibition |
|---------------------------------|-------------------|------------|------------------------|
| <i>Escherichia coli</i>         | ATCC 25922        | 7.1        | 100                    |
| <i>Staphylococcus aureus</i>    | ATCC 25923        | 7.1        | 100                    |
| <i>Streptococcus pneumoniae</i> | ATCC 49619        | 7.1        | 100                    |
| <i>Haemophilus influenzae</i>   | ATCC 49247        | 7.1        | 76                     |
| <i>Enterococcus faecalis</i>    | ATCC 29212        | 7.1        | 99.6                   |
| <i>Pseudomonas aeruginosa</i>   | ATCC27603         | 7.1        | 80                     |
| <i>Pseudomonas aeruginosa</i>   | (Multi-Resistant) | 7.1        | 86                     |
| <i>Klebsiella pneumoniae</i>    | ATCC70603         | 7.1        | 39                     |

*E. coli*, *S. aureus*, and *S. pneumoniae* growth in Luria-Bertani culture medium; *H. influenzae* and *E. faecalis* growth in BHI culture medium.

has a predominantly random conformation in aqueous environment (Table 3 and Fig. 2A, 0% TFE). BTM-P1 peptide secondary structure content (expressed as percentage) increased proportionally to the environment's hydrophobicity, where the BTM-P1 peptide preferentially adopted a helicoidal conformation (Fig. 2A) at 50% TFE, this being the highest mol. ellip. TFE's stabilising effect was suggested by TFE molecules' preference for aggregating around the peptide. Water was consequently displaced and hydrogen bridges were removed, favouring hydrogen bridge formation on the inner side of the peptide. It is known that the peptide's structure in TFE differs to that in a biological membrane's hydrophobic environment wherein the peptide interacts with lipids. BTM-P1 CD spectra were recorded in two types of liposomal membranes (Fig. 2B). BTM-P1 adopted a more  $\alpha$ -helicoidal conformation in the environment provided by 1:1:1 PE:PC:CH liposomes than in 1:1 PE:CH liposomes (Fig. 2B).

#### NMR analysis

The peptide's NOESY spectrum showed  $\alpha$ N ( $i, i+1$ ) sequence signals to be stronger than intra-residue cross-peaks. The presence of  $d_{NN}$  cross-peaks indicated a significant conformation population in the  $\alpha$  region of the  $\phi\psi$  space. Some medium-range  $d_{NN}$  ( $i, i+1$ ),  $d_{\alpha\beta}$  ( $i, i+3$ ),  $d_{\alpha N}$  ( $i, i+3$ ), and  $d_{\alpha N}$  ( $i, i+4$ ) cross-peaks were found for some amino-acids (K14–F21), suggested by lowered amide

proton chemical shift temperature coefficients, indicating the presence of a typical helical fragment in BTM-P1 peptide between residues K6–A22. The NOEs between P3  $\delta$  protons with A2  $\alpha$  proton and A2  $H^N$  proton indicated that this peptide was a *trans*-isomer. BTM-P1 sequential medium range NOEs and  $\Delta\delta H^N/\Delta T$  are summarised in Fig. 3.

#### Structure calculations

The calculations were repeated until a structure containing a minimum of distance and angle restraint violations and the least root-mean square deviation (RMSD) was obtained, respecting consensus least energy structure. Structures having reasonable geometry and few violations were then selected. A set of 50 independently produced structures were obtained from BTM-P1 peptide, satisfying the experimental constraints when using 217 distance restraints (including 102 inter-residue constraints) derived from the NOEs previously classified by signal-strength. 25  $\omega$  dihedral angle restraints were used. Two hydrogen bond constraints were introduced for K14 and F21 residues HN having  $<4$  ppb/K temperature coefficients with C=O corresponding  $i-4$  residues. A family of 13 low-energy conformers was accepted which did not have a distance violation larger than 0.3 Å. These structures had a 0.2 Å RMSD superimposition value for the backbone atoms. BTM-P1 structures were helical between residues K6–A22 (Fig. 4).

#### Discussion

Antimicrobial activity on bacteria was proved; a number of cationic peptides have shown their ability to permeabilise the inner mitochondrial membrane [22]. BTM-P1's ionophore activity on rat liver mitochondria showed some selectivity in energised mitochondria [14].

The origin of BTM-P1 from Cry11Bb1 toxin (which is active against mosquitoes) opens up the interesting possibility that some Cry toxin regions could influence mitochondrial function in mosquito cells. BTM-P1's ionophore activity on rat liver mitochondria resembled the activity of valinomycin in isotonic  $KNO_3$  medium (Fig. 1A) and the activity of FCCP in isotonic  $NH_4NO_3$  medium (Fig. 1B). BTM-P1's activity on mitochondria was interesting because it predicted antimicrobial activity.

Table 3  
Circular dichroism analysis of BTM-P1. Peptide BTM-P1 CD spectrum in medium with increasing hydrophobicity (% TFE)

| % TFE * | % secondary structure (fraction) |              |              |               | % total (fraction) |
|---------|----------------------------------|--------------|--------------|---------------|--------------------|
|         | Helix                            | Beta         | Loop         | Disordered    |                    |
| 0       | 0.0 (474.6)                      | 27.3 (474.6) | 11.4 (198.2) | 61.3 (1066.1) | 100.0 (1738.9)     |
| 10      | 19.4 (407.5)                     | 33.1 (697.3) | 0.0 (0)      | 47.5 (999.3)  | 100.0 (2104.1)     |
| 20      | 40.5 (829.9)                     | 17.6 (360.2) | 0.0 (0)      | 42.0 (861.4)  | 100.0 (2051.4)     |
| 30      | 54.6 (1456.1)                    | 9.6 (256.9)  | 0.0 (0)      | 35.7 (951.9)  | 100.0 (2664.9)     |
| 40      | 68.2 (1479.4)                    | 0.0 (0)      | 0.0 (0)      | 31.8 (688.2)  | 100.0 (2167.6)     |
| 50      | 65.2 (1455.7)                    | 0.0 (0)      | 0.0 (0)      | 34.8 (777.9)  | 100.0 (2233.5)     |

\* TFE, Trifluoroethanol diluted in pure water ( $H_2O$ ) at the indicated concentration. 0.18 M final peptide concentration.

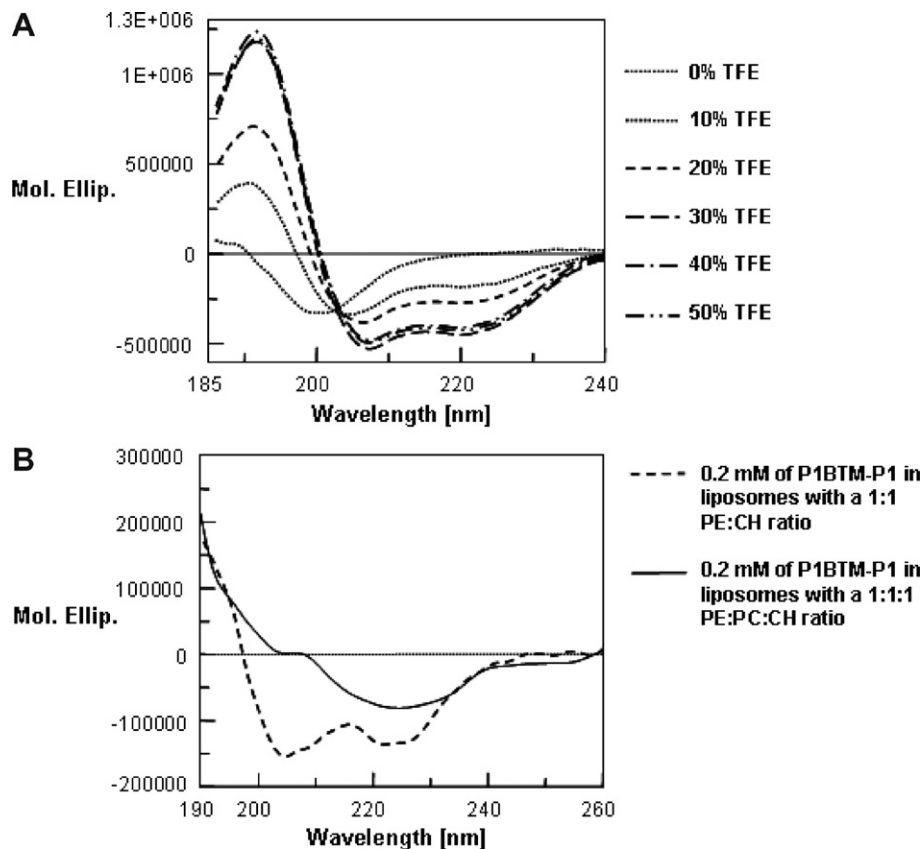


Fig. 2. BTM-P1 far-UV CD spectra. The shape of the curve in the sample shows typical  $\alpha$ -helix behaviour.

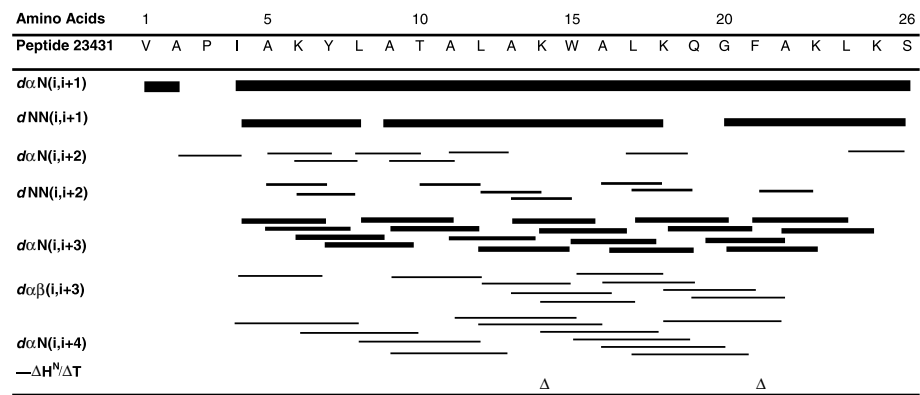


Fig. 3. Summary of sequential NOEs observed for peptide BTM-P1. The bars connect residues between which sequential NOEs were observed. The thickness of the bars indicates the NOE intensity.  $\Delta$  indicate  $H^N$  protons in H bond.

The ionophoric activity of BTM-P1 described by Lemesko et al. [13] strongly suggests that this could be the case. BTM-P1's antimicrobial activity on bacteria was clearly demonstrated in this work.

Despite Cry toxins' accepted mode of action on insect plasma membranes and its colloid osmotic lysis mechanism [11], there is evidence indicating that *B. thuringiensis* toxins can act as an uncoupler in mitochondria [23]. In fact, it has been observed that Cry toxin activity resembles that of valinomycin in insects [24] and Travers et al. [23] have demonstrated that Cry toxins could change oxygen uptake and

P/O ratio in isolated *Bombyx mori* mitochondria. More importantly, at lower toxin concentration, oxygen uptake returned to nearly normal values, whereas the P/O ratio remained essentially unchanged indicating that the toxin might act as an uncoupler.

BTM-P1 conformation with model membranes was investigated by CD with zwitterionic phosphatidylcholine–phosphatidylethanolamine–cholesterol and phosphatidylcholine–cholesterol vesicles. CD spectra data indicate that lipid vesicles were the BTM-P1 conformational factor; however, the peptides relatively higher molar ellipticity

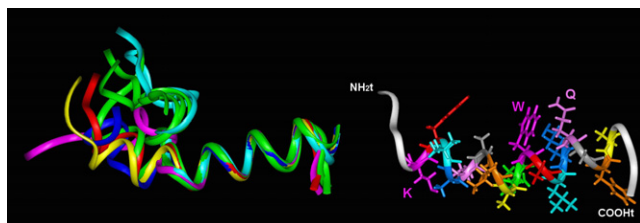


Fig. 4. BTM-P1 ribbon representation obtained by NMR. 3D structure of family of calculated structures selected for BTM-P1; each colour represents a particular calculated structure. The consensus structure is shown at right with the charged amino acids, amino (NH<sub>2</sub>) and carboxy (COOH) terminals.

trend in 1:1:1 PE:PC:CH vesicles than in 1:1 PE:CH vesicles indicated that BTM-P1 interaction with zwitterionic phosphatidylcholine mediated a better interaction.

Cationic peptide interaction with the bacterial membrane has focused on bacterial membrane phospholipids as being these peptides' main target. The inner membrane of Gram-negative bacteria and the single membrane of Gram-positive bacteria are composed of negatively charged phospholipids. By contrast, the outer surface of normal mammalian cells is predominantly assembled by zwitterionic phospholipids and cholesterol. BTM-P1 activity in both Gram positive and Gram negative bacteria may be explained by BTM-P1 entry into the zwitterionic and negatively charged membranes. Peptide net positive charge, which is antimicrobial peptides' most conserved property, allows cationic peptides to preferentially bind to the negatively charged bacterial membrane. BTM-P1 bacterial binding activity thus seems to be mainly modulated by the lipids on the bacterial membrane. In agreement with Lemeshko et al., who proposed that positively charged amino acids spreaded along the BTM-P1 chain are continuously in contact with the lipid head group during membrane permeation [13], channel formation requires peptide penetration into the membrane's hydrophobic core; then, BTM-P1 interaction with mitochondria and bacterial membranes may be predominantly governed by hydrophobic interactions, as suggested for membrane-active peptides, although it has been also proposed that neighbouring membrane electric field tension might be important for a peptide's suitable orientation to enter the membrane [13].

The increasing problem of antibiotic resistance among pathogenic bacteria requires the development of new antimicrobial agents. It has been clearly demonstrated that Gram-negative bacteria outer membranes are semipermeable molecular sieves restricting antibiotics and the access of host defence molecules to their targets within bacterial cells [3]. Thus, cations and polycations accessing the self-promoted uptake system are able to increase Gram-negative pathogenic bacteria's susceptibility to antibiotics and host defence molecules by virtue of their ability to interact with and break-down the outer membrane permeability barrier.

The non-cell-selectivity observed in the antimicrobial test thus suggests that BTM-P1 predominantly interacts with membrane phospholipids through the peptide's hydrophobic regions. Changes in the positions of some BTM-P1 hydrophobic residues and positively charged residues may thus help to increase specificity. BTM-P1's amphipatic nature was shown, this being important for activity as two truncated versions of BTM-P1 (an amino extended (P8) and truncated version (P9) charged +4) were unable to induce mitochondrial swelling.

BTM-P1's antimicrobial activity and 3-D structure have been described. Research has indicated that BTM-P1 permeabilises the mitochondrial inner membrane for cations (H<sup>+</sup>, K<sup>+</sup> and Na<sup>+</sup>), providing a plausible hypothesis explaining that the peptide's activity lies in disrupting the bacterial membrane's permeability barrier and the inner mitochondrial membrane. BTM-P1 ionophore activity has no-apparent relationship with Cry toxins permeabilising activity and it remains to be determined whether the region represented by the peptide is involved in Cry11Bb toxin pore formation. Evidence presented suggests that short Cry toxin peptides may constitute bacterial virulence factors.

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## All-D amino acid-containing channel-forming antibiotic peptides

(D enantiomeric peptides/antimalarial peptides/cecropin/magainin/melittin)

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**ABSTRACT** The D enantiomers of three naturally occurring antibiotics—cecropin A, magainin 2 amide, and melittin—were synthesized. In addition, the D enantiomers of two synthetic chimeric cecropin–melittin hybrid peptides were prepared. Each D isomer was shown by circular dichroism to be a mirror image of the corresponding L isomer in several solvent mixtures. In 20% hexafluoro-2-propanol the peptides contained 43–75%  $\alpha$ -helix. The all-D peptides were resistant to enzymatic degradation. The peptides produced single-channel conductances in planar lipid bilayers, and the D and L enantiomers caused equivalent amounts of electrical conductivity. All of the peptides were potent antibacterial agents against representative Gram-negative and Gram-positive species. The D and L enantiomers of each peptide pair were equally active, within experimental error. Sheep erythrocytes were lysed by both D- and L-melittin but not by either isomer of cecropin A, magainin 2 amide, or the hybrids cecropin A-(1–13)-melittin-(1–13)-NH<sub>2</sub> or cecropin A-(1–8)-melittin-(1–18)-NH<sub>2</sub>. The infectivity of the bloodstream form of the malaria parasite *Plasmodium falciparum* was also inhibited by the D and L hybrids. It is suggested that the mode of action of these peptides on the membranes of bacteria, erythrocytes, plasmodia, and artificial lipid bilayers may be similar and involves the formation of ion-channel pores spanning the membranes, but without specific interaction with chiral receptors or enzymes.

The cecropins (1, 2) and several other antibiotic peptides of the animal kingdom, including defensins (3), magainins (4), and the bee venom toxin melittin (5), are thought to function through the formation of ion channels in lipid membranes. This idea has been based on recent studies of electrical conductivity in artificial lipid bilayers (3, 6–8), where activity is a function of the structure of the peptide and the composition of the membrane lipids. The bilayer lipids and cell membranes are chiral and contain many asymmetric centers. It has been generally assumed that the chirality of the membrane would require a specific chirality of the peptide for it to be active, in much the same way that peptide hormones are required to fit with the conformation of their natural receptors or for a substrate and enzyme to form a tight stereospecific complex. However, we have suggested that these peptide antibiotics can exert their effect without requiring a specific target receptor on the cell membrane (7, 9).

The purpose of the present study was to test this assumption by the synthesis of the all-D enantiomers of several natural, all-L peptide antibiotics and some of their active analogs. These D stereoisomers would be expected to assume equivalent, but mirror image, conformations when placed in the same environment as the all-L peptides. If a close molecular contact with the chiral components of the cell membrane is required, the D enantiomers would be expected

to be inactive. However, if the interaction of the peptide with the membrane is only between achiral components, or if the requirement is simply for an appropriate hydrophobic environment, the D antibiotics might be expected to form active ion channels and cause the lysis and killing of cells. In addition to throwing light on its mechanism of action, such an active all-D peptide would have certain practical advantages as an antibiotic.

### MATERIALS AND METHODS

**Synthesis and Purification of the Peptides.** All of the peptides were synthesized by the solid-phase peptide synthesis technique (10, 11). They were prepared on a benzhydrylamine resin (0.65–0.9 mmol N per g), either manually by our most effective methods (12) or on an Applied Biosystems model 430A synthesizer. The couplings, with dicyclohexylcarbodiimide in dichloromethane, or symmetrical anhydrides in dimethylformamide, were monitored by the quantitative ninhydrin method (13). The *N*<sup>α</sup>-*tert*-butoxycarbonyl (Boc) group was removed from the completed peptide-resin and the peptide was deprotected and cleaved with low/high HF. It was purified on Sephadex G-25 and then on a 2.2 × 25 cm column of Vydac C<sub>18</sub> (218TPB1520) by elution with a linear gradient of 15–60% (vol/vol) acetonitrile in aqueous 0.05% F<sub>3</sub>CCOOH. The center cut of the main peak was assessed for homogeneity by analytical HPLC, and molecular weights were determined by <sup>252</sup>Cf fission fragment time-of-flight mass spectrometry.

**Circular Dichroism.** Spectra were recorded on an Aviv 62DS CD spectropolarimeter. Peptide solutions contained 25  $\mu$ M peptide and 0–20% (vol/vol) hexafluoro-2-propanol (HFP) in 2.5 mM sodium phosphate, pH 7.4. Measurements were in a 1-mm cell from 270 to 190 nm at 1-nm intervals, and three scans were averaged. The percentages of  $\alpha$ -helix,  $\beta$ -sheet, turns, and random coil were calculated by the Prose program, which is based on the data of Chen and Yang (14).

**Enzymatic Degradation.** Stock solutions of enzymes containing trypsin at 5 mg/ml or immune inhibitor A (InA) (15) at 0.1 mg/ml were diluted and mixed with an equal volume of solution containing the peptide (D-cecropin A or L-cecropin A) at 1.3  $\mu$ g/ $\mu$ l (325  $\mu$ M). All samples were incubated for 20 min at 36°C. After the incubation, 3  $\mu$ l was placed in wells on plates seeded with *Escherichia coli* D21 to determine the amount of antibiotic remaining. The diameter of the inhibition zone was compared with a previously prepared standard curve.

**Electrical Conductivity Measurements.** The equipment and procedures were similar to those described previously (7, 16). Bilayers of the Mueller–Rudin type (17) were formed from 15

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Abbreviations: Boc, *tert*-butoxycarbonyl; CA, cecropin A fragment; HFP, hexafluoro-2-propanol; InA, immune inhibitor A; LC, lethal concentration; M, melittin fragment; [Phy<sub>2</sub>]PtdCho, diphytanoyl phosphatidylcholine; PtdSer, phosphatidylserine.

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**Cecropin A:** K W K L F K K I E K V G Q N I R D G I I K A G P A V A V V G Q A T Q I A K-NH<sub>2</sub>  
**Melittin:** G I G A V L K V L T T G L P A L I S W I K R K R Q Q-NH<sub>2</sub>  
**Magainin 2 amide:** G I G K F L H S A K K F G K A F V G E I M N S-NH<sub>2</sub>  
**CA(1-13)M(1-13)NH<sub>2</sub>:** K W K L F K K I E K V G Q G I G A V L K V L T T G L-NH<sub>2</sub>  
**CA(1-8)M(1-18)NH<sub>2</sub>:** K W K L F K K I G I G A V L K V L T T G L P A L I S-NH<sub>2</sub>

FIG. 1. Amino acid sequences of some ion channel-forming antibacterial peptides. The last two are hybrids, with CA indicating cecropin A and M indicating melittin. The residue numbers (shown in parentheses) are those of the parent peptides.

mg/ml solutions of lipids in decane on a 1-mm-diameter hole in a Teflon septum separating two 1.5-ml cells. The rear (trans) compartment was held at ground and voltage was applied to the front (cis) compartment. The peptides were dissolved in 0.1 M NaCl/10 mM Hepes, pH 7.2, buffer and added to the cis compartment, final concentrations 40–4000 ng/ml.

**Inhibition Zone Assay.** Thin agarose plates were prepared as described previously (18) from 6 ml of rich medium containing  $1-4 \times 10^5$  logarithmic-phase cells of a test organism. Serially diluted peptide samples (3  $\mu$ l) were placed in 3-mm-diameter wells in the plates. After incubation for 18 hr at 30°C the diameters of the zones of inhibition around the wells were measured. The square of the diameter was plotted against the logarithm of the molar concentration of peptide, and from the slope and intercept the lethal concentration, LC, of the peptide was calculated according to ref. 19.

**Erythrocyte Lysis.** The antibacterial plate assay was adapted to an erythrocyte lysis assay. The plates contained 6 ml of 1% agarose, 0.9% NaCl, and 10% sheep erythrocytes suspended in Alsevers solution. The dilution series of peptide was placed in the wells. After incubation at 30°C for 24 hr, the diameters of the clear zones were recorded and LC values were calculated as before.

**Antiparasitic Assay.** The bloodstream form of the malaria parasite *Plasmodium falciparum* was assayed by measuring the inhibition of the reinvasion of human erythrocytes (20). Strain F32 (Tanzania) was incubated for 20 hr at 37°C in a tissue culture medium with different concentrations of the peptide. After acridine orange staining, the percentage of newly infected erythrocytes was scored in a fluorescence microscope.

## RESULTS

### Physical and Chemical Properties of the Synthetic Peptides.

The amino acid sequences of the peptides reported here are given in Fig. 1. The peptides are considered to be pure and of correct structure on the basis of analytical HPLC, mass spectrometry, and amino acid analysis.

The solution conformation was measured by circular dichroism as a function of solvent composition between 0% and 20% (vol/vol) HFP (Table 1). In aqueous solution the pep-

tides were largely random coils or  $\beta$  structures, with no observable  $\alpha$ -helix. Helicity increased with increasing HFP. Cecropin A reached 75% helix in 16% HFP, melittin was  $\approx 60\%$  helical in 4–8% HFP, while magainin 2 amide was only 44% helix in 20% HFP. CA-(1-13)-M-(1-13)NH<sub>2</sub> was strongly helical (up to 90%) above 8% HFP and CA-(1-8)-M-(1-18)NH<sub>2</sub> reached 63% helix at 20% HFP. The circular dichroism curves ( $\theta$  vs  $\lambda$ ) for every peptide pair were exact mirror images, with ellipticities equivalent but of opposite sign (Fig. 2).

The resistance of L- and D-cecropin A to enzymatic cleavage by the enzymes trypsin and InA (15) is illustrated in Fig. 3 *Upper* and *Lower*, respectively. The all-L peptide was hydrolyzed and inactivated rapidly by trypsin (50% in 20 min at a peptide-to-enzyme weight ratio of 2500:1) or by InA (50% in 20 min at a ratio of 200:1). In sharp contrast, the all-D peptide was completely stable to both enzymes, up to a concentration of trypsin 2000 times higher than needed for 50% inactivation of the L peptide. In addition, experiments in rabbit serum showed that L-cecropin A was 50% degraded in 2 hr, whereas the D enantiomer was much more stable (half-time for loss of activity, 30 hr).

**Antibacterial Activity.** The antibacterial activity of the peptides was determined by an inhibition zone assay (18) on agarose plates. Table 2 gives the LC assay data for a representative set of test organisms, which includes two Gram-negative species, *Escherichia coli* D21 and *Pseudomonas aeruginosa* OT97, and three Gram-positive species, *Bacillus subtilis* Bs11, *Staphylococcus aureus* Cowan 1, and *Streptococcus pyogenes*. Although the sensitivity of the organisms to these antibacterial peptides varied considerably, the bacteria were generally lysed by micromolar concentrations. The least sensitive of this group to cecropin A or magainin 2 amide was *Staph. aureus*, which required  $>300 \mu$ M peptide. However, as reported recently (18), the hybrid analog CA-(1-13)-M-(1-13)NH<sub>2</sub> was about two orders of magnitude more effective, and this is true also for CA-(1-8)-M-(1-18)NH<sub>2</sub>. Melittin is also much more active, but is not useful as an antibiotic because it is highly toxic to many cells, including sheep erythrocytes (last column, Table 2). In contrast, the other compounds did not significantly lyse these representative eukaryotic cells.

Table 1. Conformer composition of the synthetic peptide antibiotics calculated from circular dichroism measurements

| Peptide                                | % $\alpha$ -helix |           |           |            |            |            | % $\beta$ structures |           |           |            |            |            | % coil    |           |           |            |            |            |
|----------------------------------------|-------------------|-----------|-----------|------------|------------|------------|----------------------|-----------|-----------|------------|------------|------------|-----------|-----------|-----------|------------|------------|------------|
|                                        | 0%<br>HFP         | 4%<br>HFP | 8%<br>HFP | 12%<br>HFP | 16%<br>HFP | 20%<br>HFP | 0%<br>HFP            | 4%<br>HFP | 8%<br>HFP | 12%<br>HFP | 16%<br>HFP | 20%<br>HFP | 0%<br>HFP | 4%<br>HFP | 8%<br>HFP | 12%<br>HFP | 16%<br>HFP | 20%<br>HFP |
| L-Cecropin A                           | 0                 | 10        | 59        | 25         | 75         | 75         | 45                   | 43        | 29        | 56         | 0          | 0          | 55        | 47        | 12        | 19         | 25         | 25         |
| D-Cecropin A                           | 0                 | 0         | 52        | 35         | 72         | 75         | 38                   | 53        | 33        | 42         | 0          | 0          | 63        | 47        | 15        | 23         | 28         | 25         |
| L-[CA-(1-13)-M-(1-13)NH <sub>2</sub> ] | 0                 | 29        | 71        | 50         | 90         | 55         | 47                   | 60        | 29        | 32         | 0          | 19         | 54        | 12        | 0         | 18         | 11         | 27         |
| D-[CA-(1-13)-M-(1-13)NH <sub>2</sub> ] | 0                 | 27        | 59        | 72         | 89         | 71         | 44                   | 73        | 37        | 0          | 0          | 0          | 56        | 0         | 4         | 28         | 11         | 29         |
| L-[CA-(1-8)-M-(1-18)NH <sub>2</sub> ]  | 0                 | 40        | 42        | 35         | 56         | 63         | 55                   | 52        | 49        | 51         | 14         | 0          | 45        | 8         | 9         | 14         | 30         | 36         |
| D-[CA-(1-8)-M-(1-18)NH <sub>2</sub> ]  | 0                 | —         | 46        | 43         | 56         | 63         | 49                   | —         | 30        | 25         | 5          | 0          | 51        | —         | 23        | 32         | 38         | 37         |
| L-Magainin-NH <sub>2</sub>             | 0                 | 0         | 42        | 38         | 38         | 44         | 68                   | 68        | 47        | 44         | 41         | 31         | 32        | 32        | 11        | 19         | 21         | 25         |
| D-Magainin-NH <sub>2</sub>             | 0                 | 0         | 42        | 30         | 39         | 43         | 69                   | 69        | 40        | 52         | 35         | 31         | 31        | 31        | 19        | 19         | 26         | 26         |
| L-Melittin                             | 0                 | 74        | 61        | 75         | 75         | 75         | 50                   | 13        | 17        | 0          | 0          | 0          | 50        | 13        | 22        | 25         | 25         | 25         |
| D-Melittin                             | 0                 | 59        | 54        | 69         | 72         | 75         | 48                   | 22        | 16        | 0          | 0          | 0          | 52        | 19        | 27        | 31         | 28         | 25         |

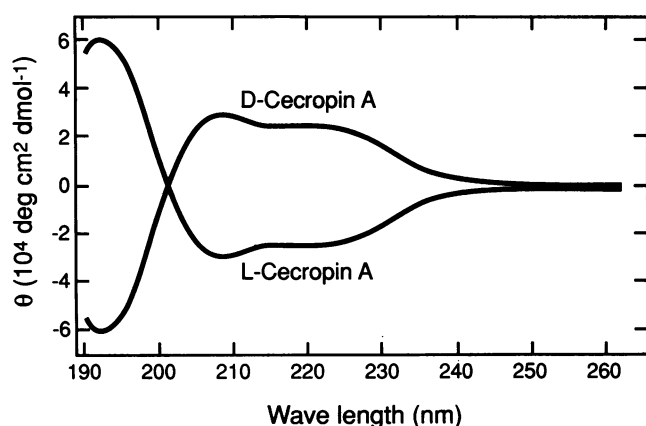


FIG. 2. Mean residue ellipticity spectra of the L and D enantiomers of cecropin A. Solvent, 20% (vol/vol) HFP in 2.5 mM sodium phosphate, pH 7.4; peptide concentration, 25  $\mu$ M; temperature, 22°C.

In all cases the activity of the D enantiomer was quantitatively equivalent to that of the L enantiomer within the limits of these experiments (approximately a factor of 2). For example, the LC ratios of the L and D isomers of cecropin A were 0.7, 1.3, 1, (1), and 2.5 for *E. coli*, *P. aeruginosa*, *B. subtilis*, *Staph. aureus*, and *Strep. pyogenes*, respectively, and neither lysed erythrocytes. For CA-(1-8)-M-(1-18)NH<sub>2</sub> the corresponding ratios were 1, 1.2, 2, 3.3, and 2.5, with no erythrocyte lysis. For melittin the ratios were 0.8, 1.5, 0.5, 2, and 0.6, and all-D-melittin lysed erythrocytes essentially as well as the L enantiomer.

**Antimalarial Activity.** Measurements of the activity of the peptides against the bloodstream form of the malaria parasite *Plasmodium falciparum* showed that the D enantiomers of CA-(1-13)-M-(1-13)NH<sub>2</sub> and CA-(1-8)-M-(1-18)NH<sub>2</sub> were 20% as active as the corresponding L isomers (50% inactivation at 10  $\mu$ M vs. 50  $\mu$ M). The two enantiomers of magainin

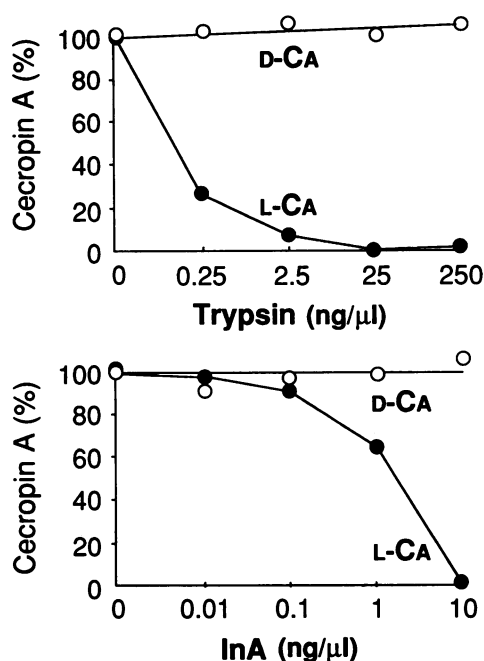


FIG. 3. Inactivation of the L and D enantiomers of cecropin A by the enzymes trypsin (Upper) and InA (Lower). Cecropin A remaining after a 20-min incubation at 36°C with enzyme was assessed by the inhibition assay on agarose plates of *E. coli* and is expressed as percent of control containing peptide but no enzyme.

2 amide were approximately one order of magnitude less active than the hybrids, and cecropin A was almost completely inactive. Melittin could not be tested because it lysed the erythrocytes.

**Electrical Conductivity in Lipid Bilayers.** Planar lipid bilayers, made of a 3:1 (vol/vol) mixture of diphytanoyl phosphatidylcholine ([Phy<sub>2</sub>]PtdCho) and phosphatidylserine (PtdSer) were tested for their conductivity as a function of voltage gradient and composition of added peptide antibiotic under conditions similar to those reported previously for natural cecropins and several of their all-L analogs (7). The D enantiomers behaved in exactly the same way as their L enantiomer controls. For example, D-cecropin A and L-cecropin A developed single-channel conductances of  $67 \pm 8$  pS and  $70 \pm 13$  pS, respectively, at -60 mV when added to the cis side of the lipid bilayer (Fig. 4), and the D and L isomers of CA-(1-13)-M-(1-13)NH<sub>2</sub> also gave equal conductances ( $130 \pm 30$  pS at -60 mV). The macroscopic conductances were voltage dependent but linear with peptide concentration (7). For cecropin A the conductance was 0.1 nS/ $\mu$ M. The details of these and similar experiments on other D and L peptide pairs will be reported elsewhere.

## DISCUSSION

An enantiomer is a molecule possessing a three-dimensional shape that is the mirror image of the parent compound. A peptide only partially substituted with stereoisomers of the component amino acids is a diastereomer but not an enantiomer. When it is composed of all-D amino acids, in the same sequence, the peptide is the D enantiomer of the all-L peptide, and it is subject to the same conformational constraints as the L enantiomer. In such a D peptide structure the normal right-handed helical segments found in the parent L peptide will be present as left-handed helices and the entire folded chains are expected to be mirror images.

There are several examples of synthetic all-D peptide hormones—e.g., bradykinin (21), oxytocin (22), and angiotensin (23). Because these hormones must interact with chiral receptors on the susceptible cell surfaces, they have, in all cases, been found to be inactive.  $\beta$ -Endorphin containing D residues in the C-terminal region, but L residues in the specific 1-5 recognition region, retained opiate activity (24). Only a few examples of synthetic enantiomers of naturally occurring peptide antibiotics are known. The earliest, to our knowledge, was enantio-enniatin B (25). However, this is a very special case, in which the high redundancy of structure of the cyclic depsipeptide allows the two isomers to become topologically superimposable simply by rotating one of the molecules by 60°. The isomer could then be recognized by the presumed chiral receptor. This work led to the idea of retroenantiomers (26), which can give peptides that are topologically superimposable with the parent isomers, but with the direction of the peptide bonds reversed, and they can retain biological activity. In these cases the objective is to make a new compound which will have the same topology as the natural derivative and will react with the same receptor. In the present work, the D isomer and the natural L isomer will not have the same topology and would not be expected to interact with a natural chiral receptor.

Anthelvencin is a recent example of an enantiomer of an antibiotic, containing a single asymmetric center, that retains the ability of the parent natural isomer to bind to the minor groove of duplex calf thymus DNA, although in an altered alignment (27). An enantiomeric peptide pair with two asymmetric centers, dihydroxybis(netropsin)succinamide, showed 10-fold differences in binding affinity in the DNA minor groove (28).

We have synthesized the L and D enantiomeric pairs of three naturally occurring peptide antibiotics—cecropin A,

Table 2. LC values for bacteria and erythrocytes

| Peptide                                | LC, $\mu$ M    |                      |                    |                      |                        | LC ratio<br>for L/D<br>isomers* | LC for<br>erythrocytes,<br>$\mu$ M |
|----------------------------------------|----------------|----------------------|--------------------|----------------------|------------------------|---------------------------------|------------------------------------|
|                                        | <i>E. coli</i> | <i>P. aeruginosa</i> | <i>B. subtilis</i> | <i>Staph. aureus</i> | <i>Strep. pyogenes</i> |                                 |                                    |
| L-Cecropin A                           | 0.2            | 1                    | 3                  | >300                 | 5                      | 1.3 $\pm$ 0.4                   | >200                               |
| D-Cecropin A                           | 0.3            | 0.8                  | 3                  | >300                 | 2                      |                                 | >300                               |
| L-Magainin-NH <sub>2</sub>             | 4              | 30                   | 3                  | 300                  | 4                      | 2.0 $\pm$ 0.9                   | 300                                |
| D-Magainin-NH <sub>2</sub>             | 1              | 30                   | 3                  | 100                  | 3                      |                                 | >400                               |
| L-Melittin                             | 0.8            | 3                    | 0.2                | 0.2                  | 0.5                    | 1.1 $\pm$ 0.5                   | 4                                  |
| D-Melittin                             | 1              | 2                    | 0.4                | 0.1                  | 0.9                    |                                 | 2                                  |
| L-[CA-(1-13)-M-(1-13)NH <sub>2</sub> ] | 0.5            | 1                    | 0.7                | 2                    | 1                      | 0.7 $\pm$ 0.2                   | >200                               |
| D-[CA-(1-13)-M-(1-13)NH <sub>2</sub> ] | 0.8            | 2                    | 1                  | 8                    | 0.8                    |                                 | 500                                |
| L-[CA-(1-8)-M-(1-18)NH <sub>2</sub> ]  | 0.3            | 0.7                  | 0.4                | 1                    | 2                      | 2.0 $\pm$ 0.5                   | >600                               |
| D-[CA-(1-8)-M-(1-18)NH <sub>2</sub> ]  | 0.3            | 0.6                  | 0.2                | 0.3                  | 0.8                    |                                 | >400                               |
|                                        |                |                      |                    |                      | Mean                   | 1.4 $\pm$ 0.5                   |                                    |

\*Data are presented as mean  $\pm$  mean deviation from the mean.

melittin, and magainin 2 amide—and two of their hybrid analogs. The peptides were all purified to apparent homogeneity and they all showed the expected amino acid composition and molecular weight by mass spectrometry. Circular dichroism measurements in solvents containing HFP showed increasing proportions of helical structure as the organic component increased. Cecropin A, for example, increased to 75% helix, which is quite close to the amount expected for a globular protein of that composition according to Chou-Fasman parameters (29). The other peptides reached 43–90% helix. The ellipticities of the D enantiomers, in every instance, were essentially equivalent to their all-L counterparts, but with opposite sign (Fig. 2). The data support the expectation that the D enantiomers do form mirror images of the L peptides.

Cecropin A was very sensitive to degradation by trypsin because of the seven L-lysine and one L-arginine residues. It was also sensitive to InA, an enzyme isolated from *Bacillus thuringiensis*, which is known to cleave cecropin A in several places (15), and furthermore it was inactivated by serum. In sharp contrast, all-D-cecropin A was very resistant to degradation by trypsin, InA, and serum. We believe this stability to be an important feature of these D antibiotics because their half-lives *in vivo* should be much longer than those of the natural peptides.

It was interesting to find that the D enantiomers of all five of the antibiotic peptides studied induced the same kinds of electrical conductivity in artificial planar lipid membranes as

their L enantiomers. Single-channel conductances were measured and there were no significant differences between the two isomers of each pair.

It has been shown (7) that the behavior of the natural cecropins on lipid bilayer membranes is well explained by the formation of electrically conducting ion channels, or pores. The pores are thought to be composed of mobile aggregates of six or more peptide chains, the largest reaching approximately 40 Å in diameter. Under these conditions, the peptides are helical and amphipathic at the N terminus and helical and hydrophobic at the C terminus (12, 30, 31). Other data (32) indicate that a flexible region is necessary near the center of peptide. We have suggested (7) that the peptides first associate with the membrane by electrostatic forces and then the helical molecules span the lipid bilayer. Association of several such peptides would form a water-filled pore, lined with many positively charged lysine residues, which would serve as an ion-conducting, anion-selective, channel.

Our interpretation of the data presented here is that the D enantiomers of these antibiotics also form ion channels, or pores, in the lipid bilayers. The results indicate that the chiral L and D isomer pairs are mirror images of one another and the helical segments may be either right-handed as in the natural all-L peptides or left-handed as in the D enantiomers. In addition, both D and L enantiomers are expected to form higher-order structures that are comparable but of opposite chirality, each maintaining an amphipathic character in which the hydrophilic charged surfaces of each helix face the interior of the water-filled pore and the hydrophobic surfaces face the lipids of the membrane. Since the behavior of the five stereoisomer pairs shows no measurable chiral selectivity, it appears that the peptides are not influenced by close interactions with any stereo centers of the membranes while exerting their effects, requiring only a hydrophobic environment to assume their opposite but equivalent final quaternary structures. Consequently, the D and L enantiomers form the same kind and number of ion channels with the same electrical conductances.

The cecropins are quite active against a wide range of Gram-negative and Gram-positive bacteria, but they do not lyse eukaryotic cells such as sheep erythrocytes and Chang liver cells (2). For many organisms they are active in the micromolar range, although some, such as *Staph. aureus*, are resistant to >300  $\mu$ M peptide. Magainin 2, a 23-residue peptide recently isolated from frog skin, has a rather similar antibacterial spectrum (4), although for some species it is severalfold less potent than cecropin A (Table 2). At high concentration magainin is measurably hemolytic, whereas cecropins are not. Melittin, the major peptide component of bee venom, is known mainly as a toxin, but it is an even more

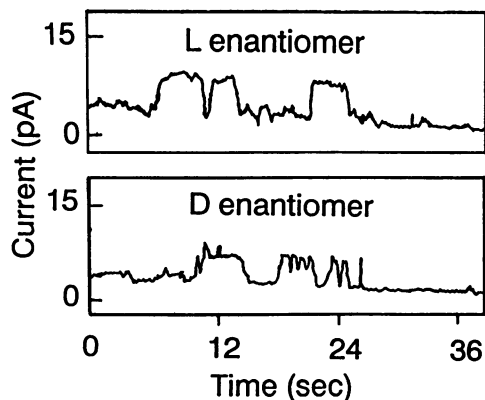


FIG. 4. Demonstration of single-channel conductance activity of the L and D enantiomers of cecropin A in lipid bilayers. Peptide concentration, 90 nM in pH 7.2 buffer, on the cis side of PtdSer/[Phy<sub>2</sub>]PtdCho membrane. Transmembrane potential, -60 mV. Start of the recordings was 30 min after addition of peptide and application of voltage gradient.

potent antibacterial peptide than cecropin A, especially toward Gram-positive bacteria. Unfortunately, the toxicity of melittin prevents its use as an antibiotic. Several of our chimeric hybrid peptides containing segments of cecropin A combined with segments of melittin were recently reported (18) to overcome the resistance of Gram-positive species such as *Staph. aureus*, and therefore they represent broader-spectrum antibiotics, which, in addition, do not lyse erythrocytes.

The striking result from our antibacterial assays was the fact that in all five isomeric pairs of peptides the activity of the D enantiomer was quantitatively equivalent to that of the L enantiomer against each of the five test organisms. The ratios of the lethal concentrations for the L and D peptides averaged  $1.4 \pm 0.5$ . Thus, there was no chiral selectivity in the lysis and killing of the bacteria. Fortunately, the great improvement in the sensitivity of *Staph. aureus* to the L hybrids, L-[CA-(1-13)-M-(1-13)NH<sub>2</sub>] and L-[CA-(1-8)-M-(1-18)NH<sub>2</sub>], was maintained by the two D analogs. Furthermore, the D enantiomers did not have increased ability to lyse erythrocytes.

It has already been shown (18) that some of these antibacterial peptides have activity against the bloodstream form of the malaria parasite *Plasmodium falciparum*. Cecropin A is only weakly active, while cecropin B and magainin 2 amide caused a 50% inhibition of the reinvasion of erythrocytes by these organisms at 100  $\mu$ M and the hybrid L-[CA-(1-13)-M-(1-13)NH<sub>2</sub>] had the same effect at only 10  $\mu$ M. Melittin itself could not be tested because it is strongly hemolytic. The D enantiomers, D-[CA-(1-13)-M-(1-13)NH<sub>2</sub>] and D-[CA-(1-8)-M-(1-18)NH<sub>2</sub>] have now been tested and were also found to be potent antibiotics against *Plasmodium falciparum* although, for this protozoan, the D enantiomers were less active than the L isomers. Presumably, these results reflect differences in the cell membranes and indicate that more than one type of cell inactivation mechanism may come into play in this complex system, one chiral and one achiral.

One of our goals has been to develop peptide antibiotics with sufficient potency and range of susceptible organisms to be of practical utility, and this has, to some extent, been achieved. The best peptides studied here, both D and L, are very active against a range of Gram-positive and Gram-negative bacteria and are much more active than either cecropin or magainin for certain species. In addition, the D enantiomers are resistant to enzymatic degradation and inactivation. Their half-lives *in vivo* should be much longer than those of the natural L forms and, as a consequence, they may be effective orally.

From all of the data obtained to date, we find that the L and D enantiomeric pairs of cecropin A, melittin, magainin 2, and two cecropin A-melittin hybrids behave the same in electrical conductance measurements in planar lipid bilayers and in antibacterial and erythrocyte assays. This suggests to us that the mechanism of action in all three systems may be similar. The simple ion-channel mechanism for the antibacterial effect of these peptides seems to fit the facts that we now have, and it is clear from the D enantiomer data that the mechanism does not involve a stereoselective interaction with a chiral enzyme or lipid or protein receptor.

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## Chapter 5.9

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# THE USE OF D-AMINO ACIDS IN PEPTIDE DESIGN

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**Keywords:** peptide hairpins,  $\alpha$ -aminoisobutyric acid, Schellman motif, ambidextrous helices, LD peptides

## 1. INTRODUCTION

Proteins and most naturally occurring peptides are composed of amino acids of the L-configuration. D-amino acids are found as constituents of natural peptides produced primarily, by microorganisms, using a non-ribosomal mechanism of synthesis. Research in this field dates back to over 60 years ago when Lipmann *et al* noted the presence of D-amino acids in tyrocidines and gramicidins [1]. Post-translational epimerization is an infrequently used mechanism in higher organisms for the introduction of D-amino acids into polypeptide chains [2]. Some notable examples are the production of opioid peptides, namely dermorphins and deltorphins from the skin of various frog species belonging to the Phyllomedusinae sub-family [3] and the contryphans produced by cone snails via post-translational epimerization [4]. Recently, total chemical synthesis of proteins containing all-D residues has been carried out and the characteristics and enzymatic activity of such enantiomeric proteins has been analyzed [5-7]. The incorporation of D-amino acids into polypeptide chains imposes local conformational constraints [8]. The purpose of this review is to examine the use of D-amino acids in the design of peptides adopting well-defined folded conformations.

## 2. CONFORMATIONAL CHARACTERISTICS OF D-AMINO ACIDS

The effects of residue configuration on polypeptide chain conformation are readily understood by considering the Ramachandran maps for N-acetyl-L-Ala-NHMe and N-acetyl-D-Ala-NHMe shown in Fig. 1A & 1B [9-12]. The map for the D-residue is derived by inverting the classical L-Ala map through the origin. Most of the stereochemically allowed regions for the L-residues correspond to negative values of  $\phi$ , while for D-residues, the allowed regions correspond to positive values of  $\phi$ . The classical  $3_{10}$  and  $\alpha$ -helical

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structures formed by all-L polypeptides adopt a right-handed twist ( $3_{10-R}/\alpha_R$ ), while the corresponding D-polypeptides form helices with a left-handed twist ( $3_{10-L}/\alpha_L$ ). Notably, there are small sterically allowed regions, which correspond to positive  $\phi$  values for L-residues and negative  $\phi$  values for D-residues. L-Proline is the most constrained of the amino acids occurring in proteins. The restraints imposed by the formation of the pyrrolidine ring restrict the allowed range of  $\phi$  values in L-Pro to  $-60 \pm 20^\circ$  (Fig. 1C) [11,12]. By extension, the allowed values for D-Pro are  $+60 \pm 20^\circ$  (Fig. 1D). D-Pro can be used to advantage in restricting the local conformation of peptide chains, facilitating the formation of prime  $\beta$ -turns. The only achiral amino acid occurring in proteins is glycine, a residue for which considerably larger regions of  $\phi$ - $\psi$  space are sterically allowed, as a consequence of the absence of a substituent at the  $C^\alpha$  atom (Fig. 2A). The Gly residue can adopt conformations on either side of the  $\phi$ - $\psi$  map. The observed distribution of Gly residues in proteins (Fig. 2B) establishes an approximately symmetric distribution about the origin [13].  $\alpha$ -Aminoisobutyric acid (Aib) is an achiral residue, which contains a pair of methyl groups at the  $C^\alpha$  carbon atom. This residue may be formally viewed as a combination of L-Ala and D-Ala residues. The stereochemically allowed regions of Ramachandran space for Aib are readily derived by considering only the regions of overlap between the L-Ala and D-Ala  $\phi$ - $\psi$  maps (Fig. 2C). Clearly, the Aib residue is largely limited to the right ( $\alpha_R$ ) and left ( $\alpha_L$ ) handed helical structures. The observed distribution of crystallographically obtained conformations for Aib residues in peptides provides overwhelming support for this expectation (Fig. 2D) [11,12]. Achiral amino acids can, therefore, also be viewed as surrogate D-amino acids in peptide design because they can adopt conformations on both sides of the  $\phi$ - $\psi$  map. While the achiral Gly and Aib residues can support conformations with both positive and negative values of  $\phi$ , D-amino acids may be used in design when positive  $\phi$  values are required to form a desired structure.

### 3. $\beta$ -HAIRPIN DESIGN

$\beta$ -Hairpins are widely present in proteins with antiparallel  $\beta$ -sheet formation being facilitated by a nucleating reverse turn. Two-residue  $\beta$ -turns belonging to the type II' and I' category are most often observed as nucleating features for hairpins in proteins [14]. In the prime turns, the residue at position  $i+1$  necessarily adopts the conformation of  $\phi \sim +60^\circ$ , while  $\psi$  values of  $\sim -120^\circ$  and  $\sim +30^\circ$  are observed for type II' and I' turns, respectively [15]. Fig.3A illustrates a view of an ideal hairpin formed by a centrally positioned nucleating type II'  $\beta$ -turn. When the stereochemistry of the  $\beta$ -turn is type II ( $\phi_{i+1} = -60^\circ$ ,  $\psi_{i+1} = +120^\circ$ ), registry of the antiparallel strands is not readily achieved. Although chain reversal occurs, the antiparallel strands drift away from one another (Fig. 3B).

In the design of  $\beta$ -hairpins,  $^D\text{Pro-Xxx}$  sequences can provide a site for nucleation of prime turns. Indeed, insertion of  $^D\text{Pro}$  residues has proved to be a successful strategy for peptide hairpin design [8,11,12,16-21]. Fig. 4 illustrates the superposition of the crystal structures of six designed peptide hairpins containing a central  $^D\text{Pro-Xxx}$  type II'  $\beta$ -turn.  $^D\text{Pro}$  residues provide a strong conformational determinant, when inserted into L-amino acid sequences. In ongoing studies, centrally positioned  $^D\text{Pro-Gly}$  sequences have been used to generate well-defined  $\beta$ -hairpin scaffolds which permit spectroscopic probing of cross-strand interactions between aromatic side chains (Fig. 5A & 5B) [22]. Multiple  $^D\text{Pro-Xxx}$  segments may be used to nucleate multistranded antiparallel  $\beta$ -sheet structures as exemplified by the design of three, four and five stranded  $\beta$ -sheets (Fig. 5C & 5D) [23-26].

In principle, hairpin nucleation may be achieved by using sequences with a high propensity to form prime  $\beta$ -turns. Specifically, the Aib- $^D\text{Ala}$  segment has been used to nucleate the formation of a  $\beta$ -hairpin in the octapeptide Boc-Leu-Phe-Val-Aib- $^D\text{Ala}$ -Leu-Phe-Val-OMe (Fig. 6A) [27]. In this case, crystallographic studies establish that the Aib- $^D\text{Ala}$   $\beta$ -turn adopts a type I' conformation, undoubtedly as a consequence of the tendency of the Aib residue to form  $\alpha_R$  or  $\alpha_L$  conformations. Thus, D-amino acid containing dipeptide segments may be used to generate both type II' and I' structures. While both turn

types can sustain  $\beta$ -hairpin formation, the orientations of the strands are significantly different, as illustrated in Fig. 6C & 6D.

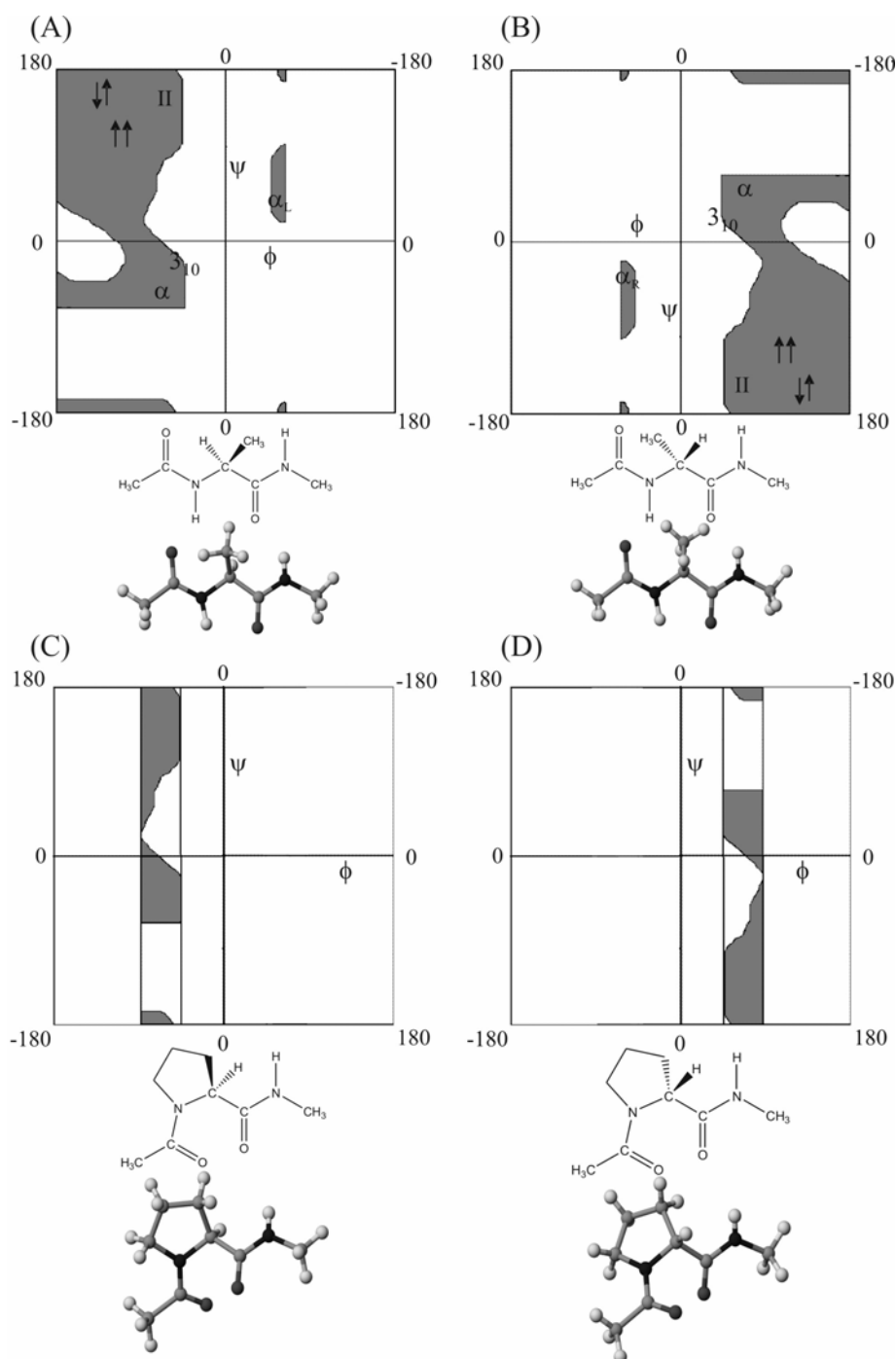


Figure 1. Ramachandran maps showing allowed regions for (A) L-Ala (B) D-Ala (C) L-Pro (D) D-Pro residues, respectively. The chemical formula and a ball-and-stick notation of N-acetyl-amino acyl-NHMe (fully extended conformation) for each amino acid are also shown below their respective maps. Figures have been adapted from [11]. Map in Figure (A) reprinted with permission from [11]; Copyright (2001) American Chemical Society.

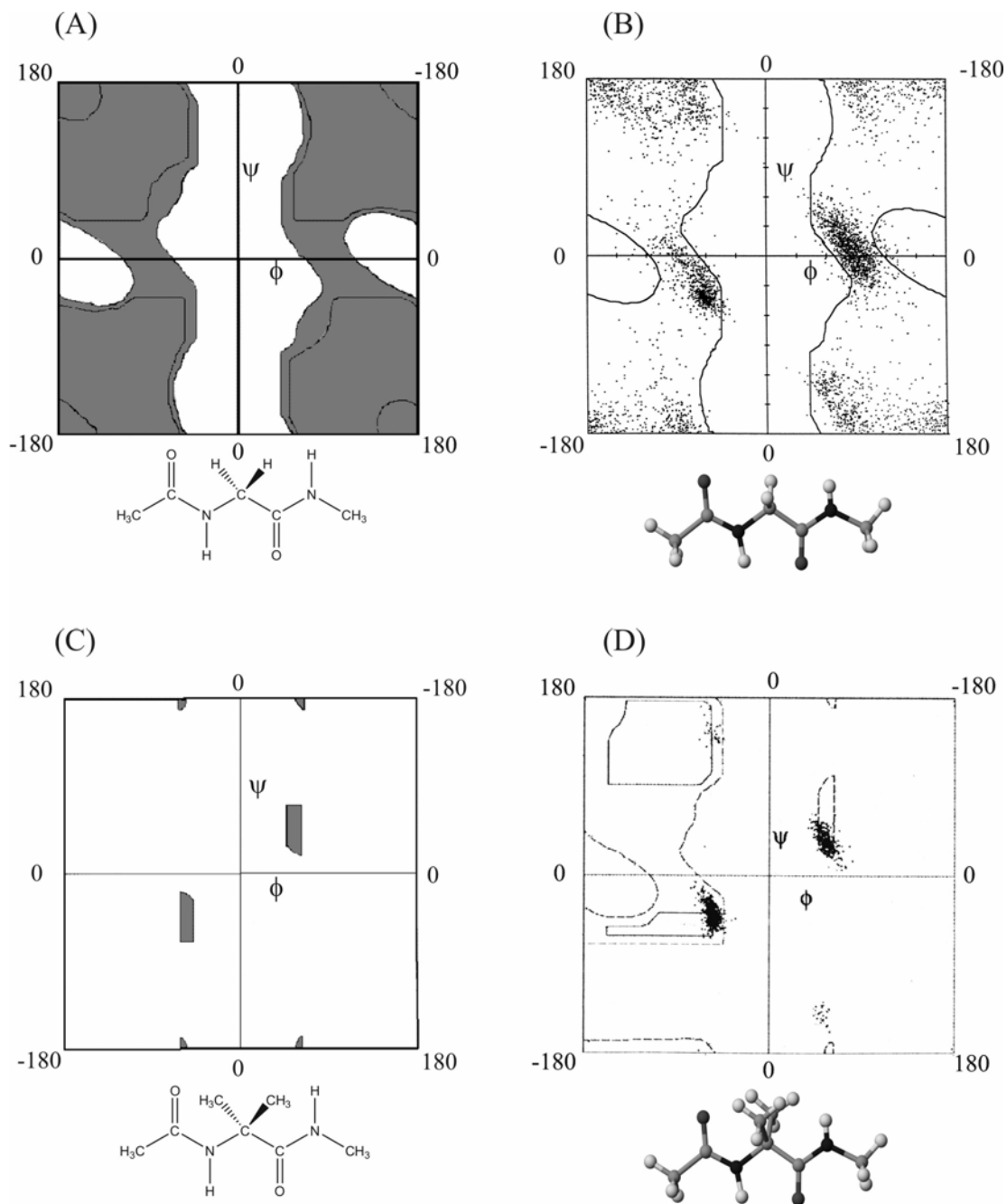


Figure 2. Allowed regions in the Ramachandran plot for Gly (A) and Aib (C) residues compared with  $\phi$ - $\psi$  values obtained from crystal structures of proteins (B) for Gly and peptides (D) for Aib. Also indicated are the chemical structures and ball-and-stick notations for these residues. Figures (A) and (C) reprinted with permission from [11]; Copyright (2001) American Chemical Society. Figures (B) and (D) reprinted with permission from [12]; Copyright (2003) Indian Academy of Sciences.

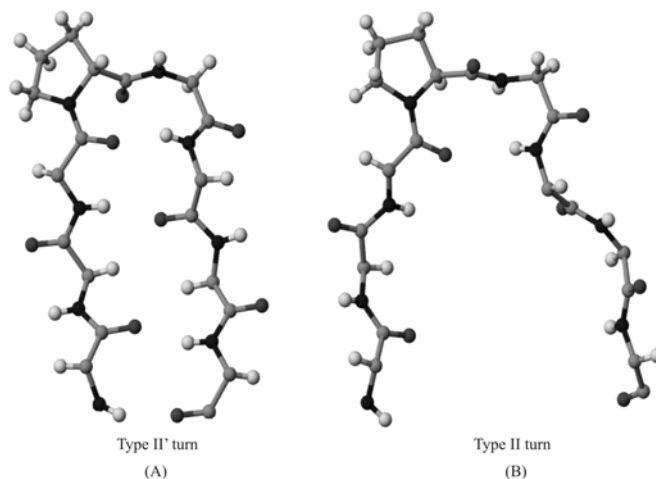


Figure 3. (A) Peptide hairpin with a type II' turn formed using  $^D\text{Pro-Gly}$  as a turn nucleator. Shown is the crystal structure of the peptide Boc-Leu-Val-Val- $^D\text{Pro-Gly}$ -Leu-Val-Val-OMe [17]. Only the peptide backbone and pyrrolidine ring of  $^D\text{Pro}$  residue are shown. (B)  $^D\text{Pro}$  in the previous peptide was replaced by  $^L\text{Pro}$  and the  $\phi$ - $\psi$  values of the turn segment were altered to ideal type II values. Although turn nucleation does occur, it is evident that the two strand segments fray away from each other. This clearly establishes the superiority of  $^D\text{Pro}$  to nucleate tight  $\beta$ -turns compared to its L-analog.

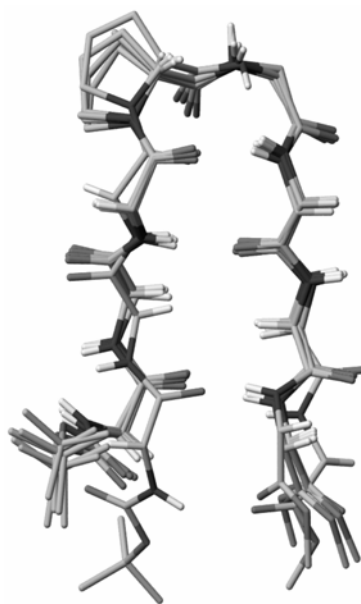


Figure 4. Superposition of crystal structures of six peptide hairpins obtained using a nucleating  $^D\text{Pro-Xxx}$  segment. All the peptides shown, form type II' turns. Sequences: Boc-Leu-Val-Val- $^D\text{Pro-Gly}$ -Leu-Val-Val-OMe [17], Boc-Leu-Val-Val- $^D\text{Pro-Ala}$ -Leu-Val-Val-OMe (2 molecules) [20], Boc-Leu-Phe-Val- $^D\text{Pro-Ala}$ -Leu-Phe-Val-OMe [21], Boc-Leu-Val-Val- $^D\text{Pro-Gly}$ -Leu-Phe-Val-OMe [21], Boc-Leu-Val-Val- $^D\text{Pro-Aib}$ -Leu-Val-Val-OMe [21]. These crystal structures provide experimental evidence for the turn-nucleating ability of  $^D\text{Pro-Xxx}$  segments, which subsequently propagates to well-registered strand segments.

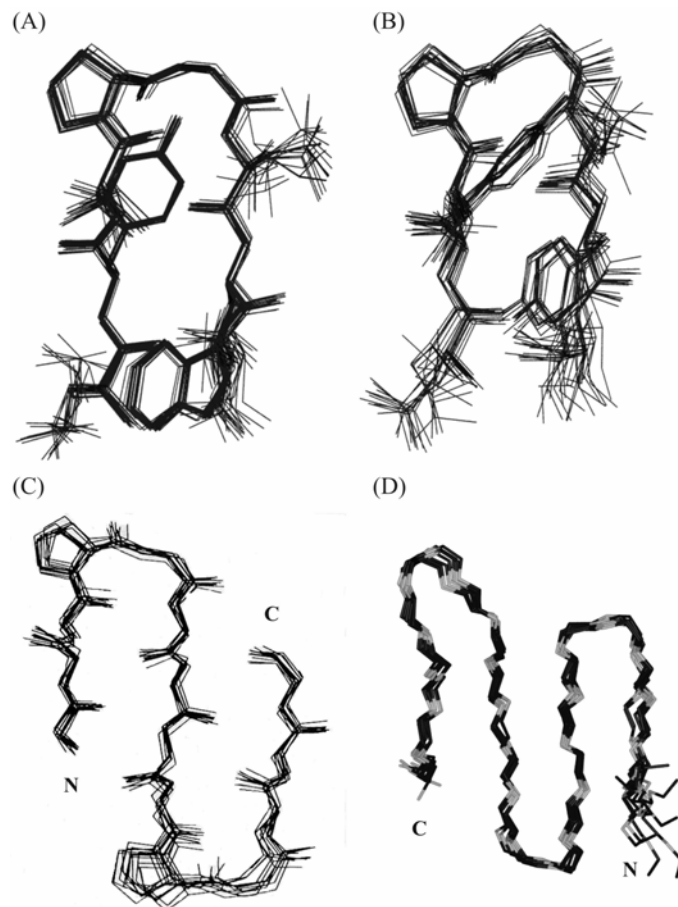


Figure 5. (A and B) Solution NMR structures of octapeptide hairpins formed using <sup>D</sup>Pro-Gly as a turn nucleator [22]. The tight turn forming ability of <sup>D</sup>Pro-Gly segments can be exploited for the construction of predefined hairpin scaffolds, which can then be made use of, for the study of tertiary interactions, such as aromatic interactions, in solution, as is demonstrated in these examples. (C and D) The ability of <sup>D</sup>Pro-Xxx segments to nucleate tight turns can be exploited for the construction of multistranded β-sheet structures. Shown are the solution NMR structures of a 14-residue three-stranded β-sheet [23] and a 26-residue four-stranded β-sheet [24]. Figures C and D reprinted with permission from [11]; Copyright (2001) American Chemical Society.

#### 4. D-AMINO ACID AS HELIX TERMINATION SIGNALS

Polypeptide helices in proteins are characterized by a succession of residues, which adopt  $\phi$ - $\psi$  values that lie in the right handed helical ( $\alpha_R$ ) region of the Ramachandran map. Helix termination at the N- and C-terminal ends occurs when residues drift away to other regions of  $\phi$ - $\psi$  space, most notably, the extended ( $\beta$ -sheet ( $\beta$ )/ polyproline ( $P_{II}$ )) and left handed helical ( $\alpha_L$ ) regions (Fig. 1A). In proteins, a widely observed structural feature at the C-terminal end of helices is the Schellman motif, which is formed when the helix terminating residue adopts an  $\alpha_L$  conformation [28,29]. In proteins structures this mode of termination predominantly involves the occurrence of Gly or Asn residues at the C-terminal position. This is a consequence of the fact that the achiral Gly residue can be readily accommodated in  $\alpha_L$  conformations, while Asn has the greatest  $\alpha_L$  propensity of the remaining chiral residues [30]. The sequence of residue conformations  $\alpha_R \alpha_R \alpha_R \alpha_R \alpha_L$  leads to helix termination with the simultaneous formation of a pair of 4→1 and 6→1 hydrogen bonds (Fig. 7A).

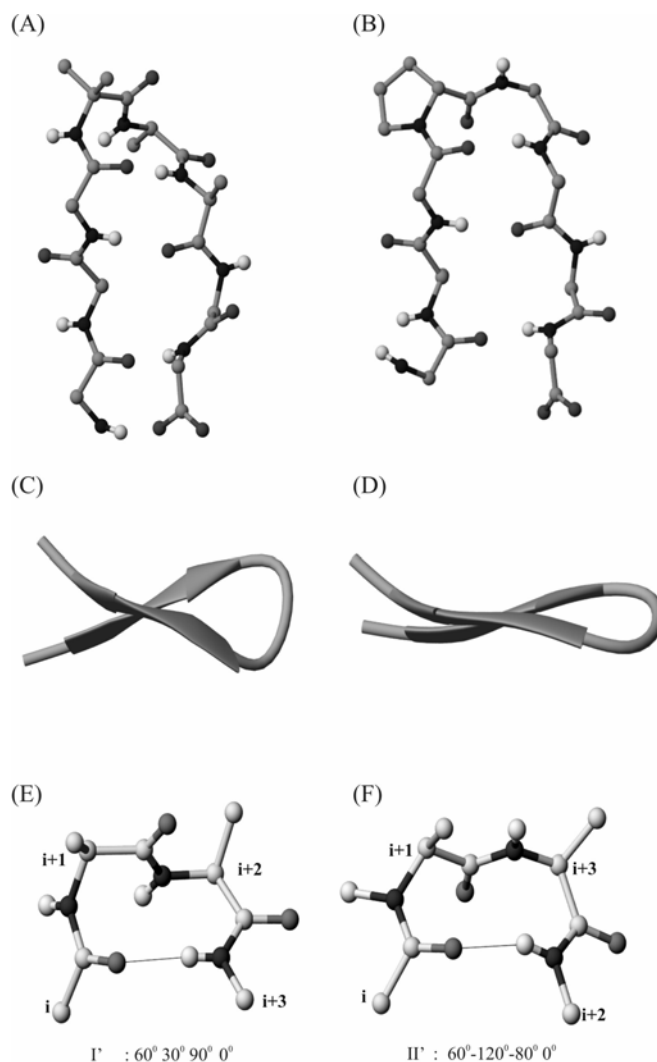


Figure 6. (A) Peptide hairpin nucleated by Aib-<sup>D</sup>Ala segment (Boc-Leu-Phe-Val-Aib-<sup>D</sup>Ala-Leu-Phe-Val-OMe) (type I' turn) [27] compared with a peptide hairpin formed using <sup>D</sup>Pro-Gly (Boc-Leu-Val-Val-<sup>D</sup>Pro-Gly-Leu-Val-Val-OMe) (type II' turn) [17] (B). Side chains of only the turn residues have been included in the figure. (C and D) Strand orientations in both peptides are however different, as illustrated by a side view of the ribbon diagram of the two hairpins. Also shown are ball-and-stick notations of the two turn types: I' (E) and II' (F).

In synthetic peptides, helix termination by a Schellman motif can be readily achieved by placement of an achiral residue or a D-residue towards the C-terminal of a potentially helical segment consisting of L-amino acids. Crystallographic characterization of Schellman motifs in peptides have been achieved in sequences containing the achiral Aib residue at the penultimate position from the C-terminus [31]. In the heptapeptide Boc-Leu-Aib-Val-Ala-Leu-Aib-Val-OMe, the molecule forms a right handed helix over the segment residues 1-5. Aib6 adopts an  $\alpha_L$  conformation resulting in helix termination (Fig. 7B) [32]. Extension of this sequence to the level of a 10-residue peptide by addition of the tripeptide sequence <sup>D</sup>Ala-<sup>D</sup>Leu-Aib-OMe results in an extension of the N-terminal helical segment with termination occurring by Schellman motif formation with <sup>D</sup>Ala8 adopting an  $\alpha_L$  conformation (Fig. 7C) [33]. This example illustrates the energetic advantage of propagating the helix with concomitant formation of intramolecular hydrogen bonds and the propensity of the <sup>D</sup>Ala residue to adopt  $\alpha_L$  conformation. Unexpectedly, the structure of this peptide also revealed an interesting chain reversal stabilized by the formation of a C-H...O hydrogen bond between the Ala4 C $\alpha$ H and <sup>D</sup>Leu9 C=O groups. This structural feature is a consequence of

the extended conformation at the  $^D\text{Leu}9$  residue with a positive value of  $\phi$  and a negative value of  $\psi$ . The generality of this structural feature has been confirmed in the crystal structure of the decapeptide analog, where Ala4 is replaced by Gly. Most importantly, replacement of  $^L\text{Ala}4$  by  $^D\text{Ala}4$  or Aib4 results in a completely different peptide conformation, establishing the importance of the C-H...O interaction in stabilizing this conformational feature [34]. A similar structural motif has also been identified in proteins [35].

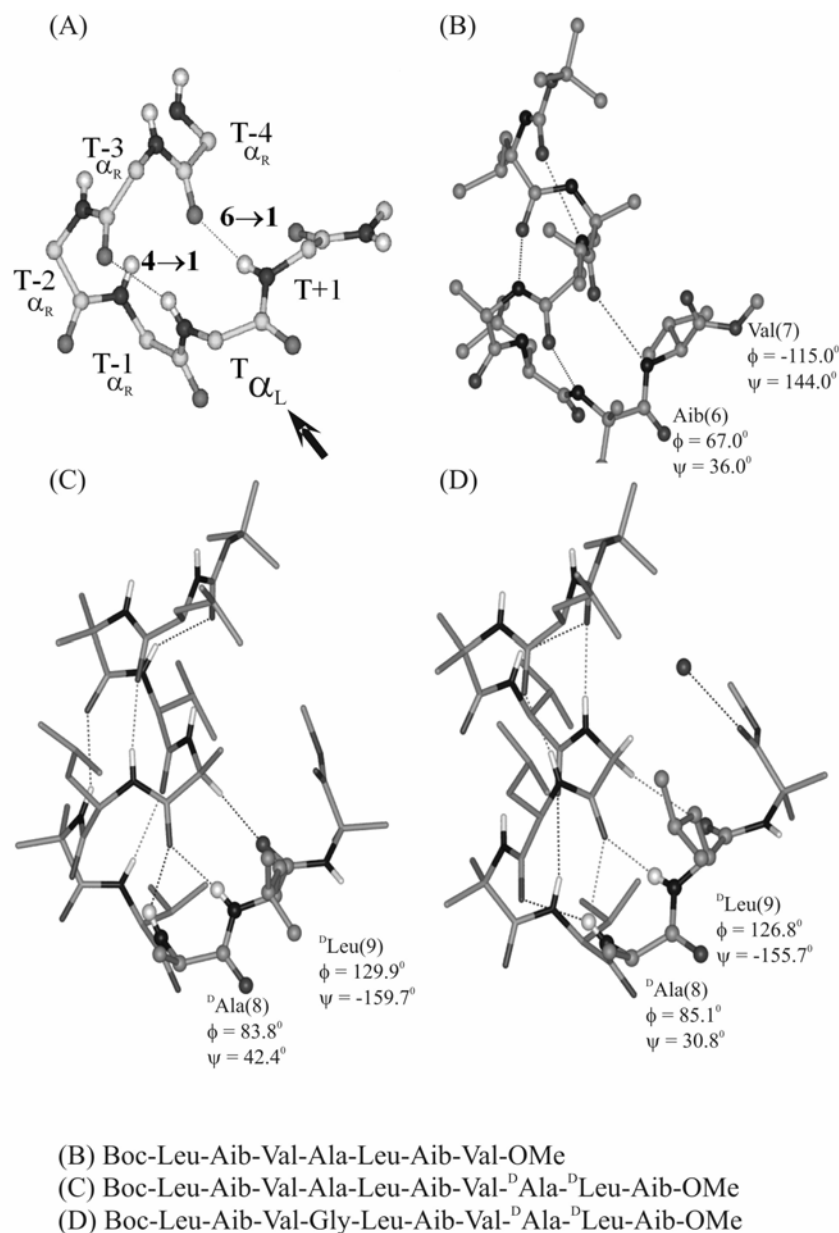


Figure 7. (A) Helix-terminating Schellman motif obtained when the terminal residue (T) adopts an  $\alpha_L$  conformation (indicated by an arrow). A 4→1 and 6→1 hydrogen bond formation stabilizes this motif [32]. (B) Crystal structure of a heptapeptide forming the Schellman motif with Aib6 adopting an  $\alpha_L$  conformation [32]. (C and D) Decapeptides containing D-amino acid residues, which function to signal helix termination [33, 34]. Figures (A), (B), (D) reprinted with permission from [12]; Copyright Indian Academy of Sciences. Figure (C) reprinted with permission from [34]; Copyright (2003) American Chemical Society.

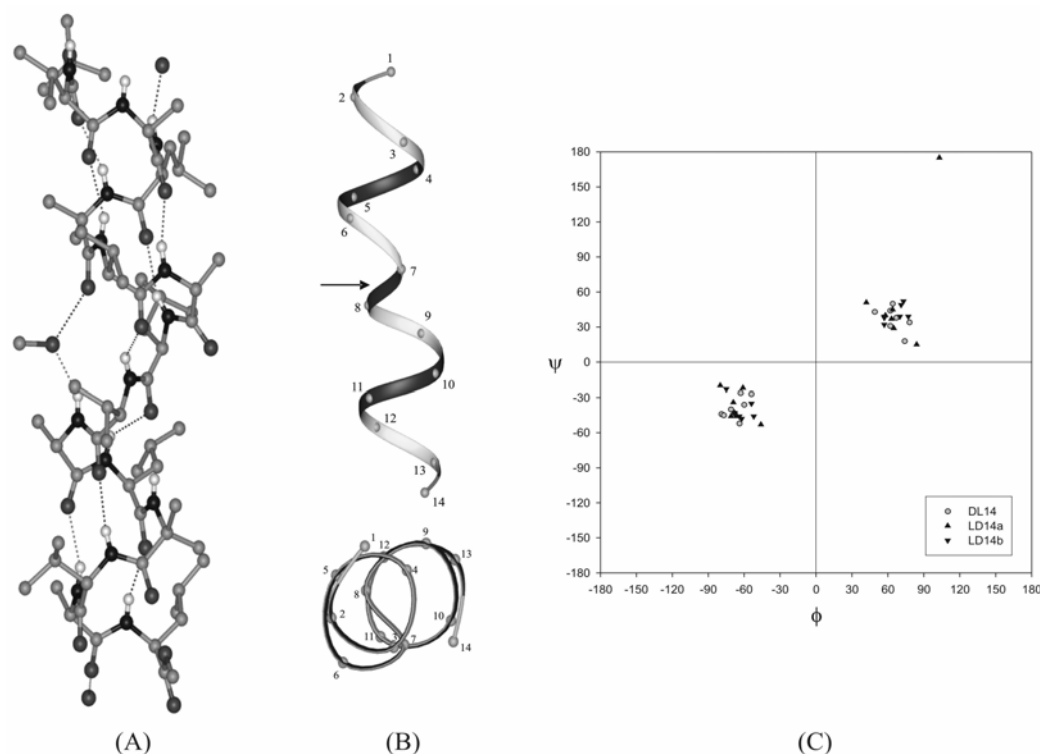


Figure 8. Ambidextrous helices formed by fusing left- and right-handed helical segments. (A) Ball-and-stick notation of a 14-residue peptide Boc-<sup>D</sup>Val-<sup>D</sup>Ala-<sup>D</sup>Leu-Aib-<sup>D</sup>Val-<sup>D</sup>Ala-<sup>D</sup>Leu-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe (DL14) [36]. The DL junction, indicated by an arrow bears the Schellman motif. (B) Ribbon diagram of the peptide viewed along the helix axis and down the helix axis. (C) Plot of the  $\phi$ - $\psi$  values obtained from the crystal structures of two ambidextrous helices. One of the peptides (Boc-Val-Ala-Leu-Aib-Val-Ala-Leu-<sup>D</sup>Val-<sup>D</sup>Ala-<sup>D</sup>Leu-Aib-<sup>D</sup>Val-<sup>D</sup>Ala-<sup>D</sup>Leu-OMe, LD14) crystallized in two polymorphic forms [37]. Figures (A) and (B) reprinted from [12]; Copyright (2003) Indian Academy of Sciences.

The ability of D-amino acid residues to facilitate Schellman motif mediated helix termination may be used to advantage in creating ambidextrous structures, which consist of directly fused right-handed and left-handed helical segments. Fig. 8 illustrates the structure of a 14-residue peptide (Boc-<sup>D</sup>Val-<sup>D</sup>Ala-<sup>D</sup>Leu-Aib-<sup>D</sup>Val-<sup>D</sup>Ala-<sup>D</sup>Leu-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe) (DL14), which consists of two distinct heptapeptide segments containing amino acid sequences of opposite chirality [36]. The achiral Aib residue at positions 4 and 11 in the sequence nucleates helix formation with the handedness of the helix being determined by the configuration of the flanking amino acid sequences. Careful inspection of the crystal structure in Fig. 8 reveals that the molecule is composed of two distinct helical segments; a left-handed helical segment for residues 1-7 and a right handed helical segment for residues 8-14. Residue 8, which adopts an  $\alpha_R$  conformation, acts as the helix terminating residue with respect to the N-terminal *left handed* helical ( $\alpha_L$ ) segment. The Schellman motif is distorted by the insertion of a methanol molecule into the 6→1 hydrogen bond. The robustness of this fold is further confirmed by the crystal structure of the enantiomeric peptide (Boc-Val-Ala-Leu-Aib-Val-Ala-Leu-<sup>D</sup>Val-<sup>D</sup>Ala-<sup>D</sup>Leu-Aib-<sup>D</sup>Val-<sup>D</sup>Ala-<sup>D</sup>Leu-OMe) (LD14), which curiously crystallized in a polymorphic form containing two molecules in the crystallographic asymmetric unit [37]. Comparison of the  $\phi$ - $\psi$  values (Table AII of [37]) confirms the similarity of the folding pattern of these sequences containing separate blocks of L- and D-amino acids.

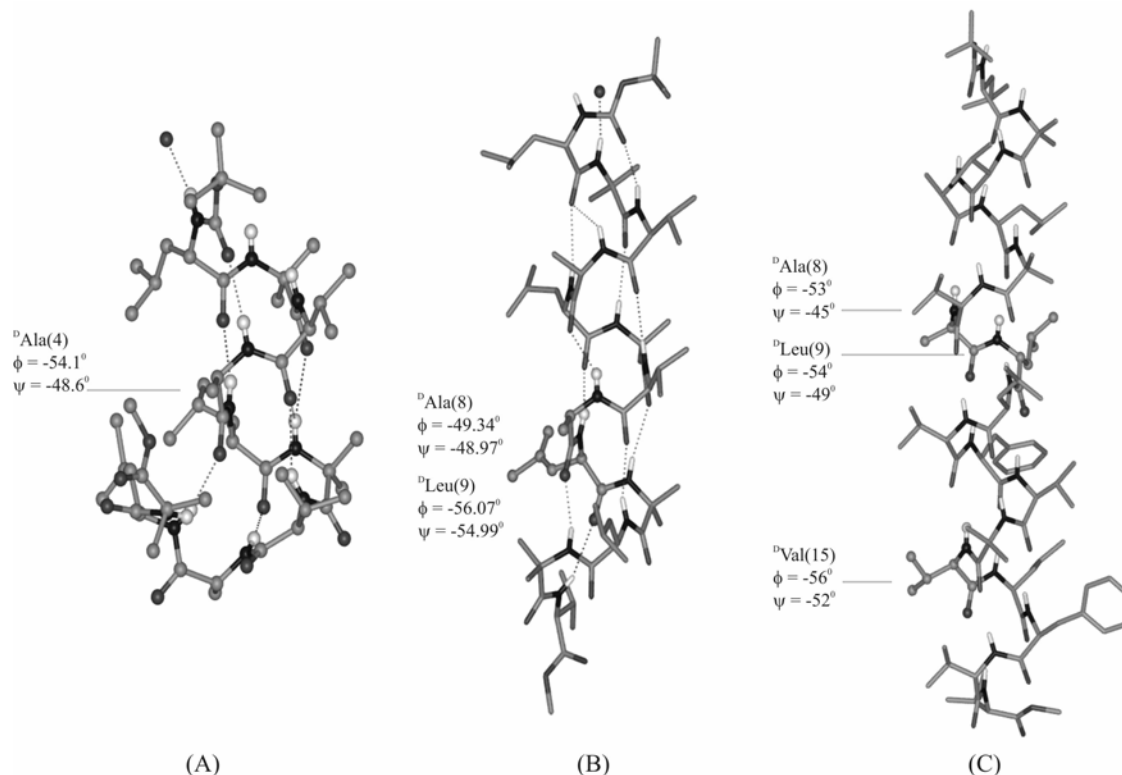


Figure 9. Examples of peptide helices containing D-amino acids incorporated in the right handed helical scaffold. (A) One D-amino acid in the right-handed helix (Boc-Leu-Aib-Val-<sup>D</sup>Ala-Leu-Aib-Val-<sup>D</sup>Ala-<sup>D</sup>Leu-Aib-OMe) [34]. (B) Two D-amino acids in the right-handed helix (Boc-Leu-Aib-Val-Ala-Leu-Aib-Val-<sup>D</sup>Ala-<sup>D</sup>Leu-Aib-Leu-Aib-Val-OMe) [40]. (C) Three D-amino acids in the right-handed helix (Boc-Leu-Aib-Val-Ala-Leu-Aib-Val-<sup>D</sup>Ala-<sup>D</sup>Leu-Leu-Val-Phe-Val-Aib-<sup>D</sup>Val-Leu-Phe-Val-Val-OMe) [41]. Figures (A) and (B) reprinted from [12]; Copyright (2003) Indian Academy of Sciences.

## 5. GUEST D-AMINO ACIDS IN RIGHT HANDED HELICAL SEGMENTS

In the synthesis of analogs of biologically active peptides, it is often desirable to introduce D-amino acids into host sequences composed of L-amino acids. The site of D-amino acid insertion becomes resistant to proteolytic cleavage, thereby resulting in greater *in vivo* stability of such analogs [38]. In designing analogs, it is necessary to retain the conformational features, which are critical for biological activity. Thus, the site of D-amino acid replacement must be carefully chosen in order to minimize the possibility of major conformational changes. The helix is one of the two most widely distributed secondary structures in peptides and proteins. It is therefore instructive to examine the consequences of inserting D-amino acids into the center of potential helical sequences. Inspection of the conformational maps in Fig. 1B clearly establishes that there is a small 'allowed window' for D-amino acids in the  $\alpha_R$  region of conformational space, suggesting that, in principle, D-amino acids can be accommodated into *right-handed* helical segments. The relatively small energy penalty for adopting positive  $\phi$  values can be readily offset by compensating interactions. Fairman *et al* have estimated the helix-forming propensity of D-residues in right handed  $\alpha$ -helices and find a penalty of 0.95kcal/mol for D-Ala when compared to the L-Ala standard [39]. Fig. 9 shows examples of synthetic peptide helices in which D-amino acids have been incorporated without any disruption of the right handed twist of the polypeptide chain. In the decapeptide (Boc-Leu-Aib-Val-<sup>D</sup>Ala-Leu-Aib-Val-<sup>D</sup>Ala-<sup>D</sup>Leu-Aib-OMe), the <sup>D</sup>Ala residue at position 4 adopts an  $\alpha_R$  conformation and is

incorporated into the right-handed helix formed by the segments 1-7. In this case, the observed  $\phi$ - $\psi$  values for the  $^D\text{Ala}$  residue in the two crystallographically independent molecules are -46.6, -52.9 and -54.1, -48.6 respectively [34]. The 13-residue peptide (Boc-Leu-Aib-Val-Ala-Leu-Aib-Val- $^D\text{Ala}$ - $^D\text{Leu}$ -Aib-Leu-Aib-Val-OMe) provides an interesting example of a double D-segment ( $^D\text{Ala}$ 8,  $^D\text{Leu}$ 9) incorporated into a right-handed helix [40]. The crystal structure of the 19-residue peptide (Boc-Leu-Aib-Val-Ala-Leu-Aib-Val- $^D\text{Ala}$ - $^D\text{Leu}$ -Leu-Val-Phe-Val-Aib- $^D\text{Val}$ -Leu-Phe-Val-Val-OMe) is a dramatic example of the accommodation of as many as three D-amino acids into a right-handed helical scaffold [41]. These examples clearly emphasize the fact that right-handed helices can readily accommodate D-residues. Selective D-amino acid substitution in strongly helical sequences may provide a convenient means of enhancing proteolytic stability, without significant disruption of overall secondary structure.

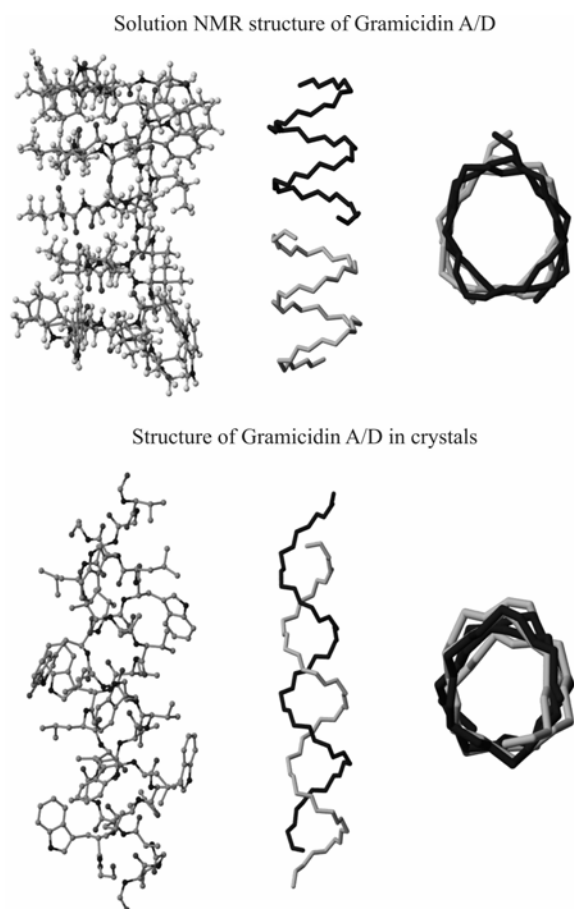


Figure 10. LD peptide helices of gramicidin. Sequence: For- $^L\text{Val}$ -Gly- $^L\text{Ala}$ - $^D\text{Leu}$ - $^L\text{Ala}$ - $^D\text{Val}$ - $^L\text{Val}$ - $^D\text{Val}$ - $^L\text{Trp}$ - $^D\text{Leu}$ - $^L\text{Trp}$ - $^D\text{Leu}$ - $^L\text{Trp}$ -Eta. Shown are the NMR- and X-ray derived structures of gramicidin A/D (PDB coordinates 1JNO [45] and 1ALZ [47], respectively). Note that although the gramicidin structures have been named 'A' and 'D', their sequences are essentially the same. Interestingly, the NMR structure reveals a  $\pi_{LD}$  helical structure for the peptide, whereas the crystal structure is a double helix.

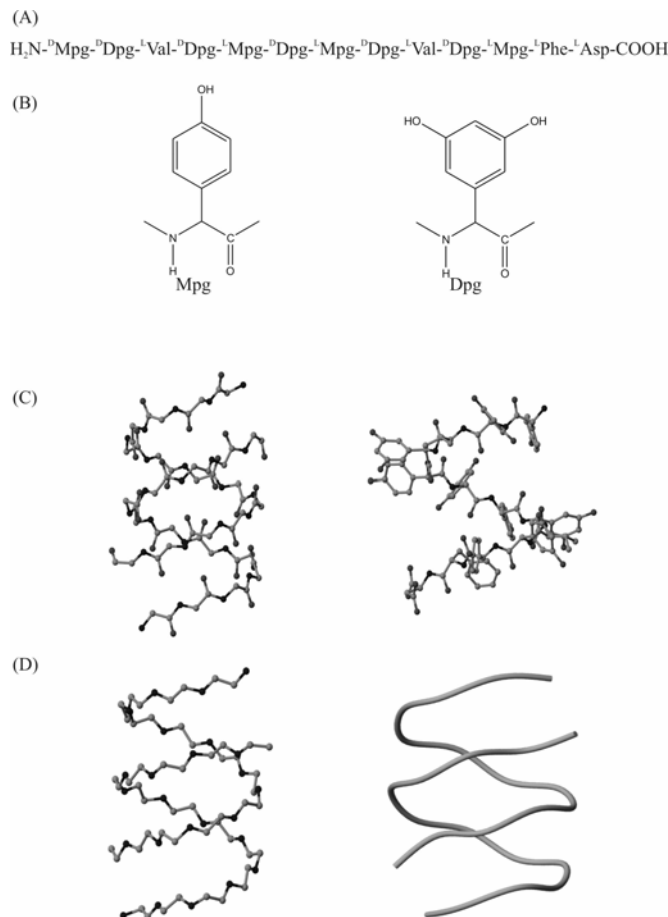


Figure 11. Feglymycin (A) sequence (B) chemical structure of the unnatural amino acids (C) different views of the double helix. Also shown is the ball-and-stick notation of the single molecule. PDB code: 1W7Q [48].

## 6. ALTERNATING LD SEQUENCES

Any discussion of the conformational effects of D-residues in peptide sequences will be incomplete without a consideration of LD polypeptides. Regular alternation of configuration is observed in the linear gramicidins, which are membrane channel forming polypeptides produced by *Bacillus brevis*. Early theoretical studies suggested that alternating LD sequences would adopt wide-bore helical structures in which an approximately extended conformation of the polypeptide was wound around a helical axis. The  $\pi_{\text{LD}}$  ( $\text{LD}_n$  helices, where  $n=3,4,5$ ) helices so generated may be viewed as helices formed by repeating LD dipeptides, in which the two residues adopt  $\beta$ -sheet conformations that lie on opposite sides of the  $\phi$ - $\psi$  map. The helices so formed are stabilized by parallel  $\beta$ -sheet hydrogen bonds between segments of the polypeptide chain on adjacent helical turns [42]. The NMR study of Gramicidin A provides confirmatory evidence for the  $\pi_{\text{LD}}$  helix (Fig. 10A) [43-45]. In crystals, an interesting variant of the  $\beta$ -helical conformation is observed with two antiparallel chains forming a double helical structure (Fig. 10B) [46, 47]. The unique structures formed by alternating LD sequences are also highlighted by a recent structure determination of the 13-residue peptide Feglymycin [48]. In this case also, an antiparallel double helical structure is formed (Fig. 11). Fig. 12 summarizes the distribution of  $\phi$ - $\psi$  values observed for the alternating LD segments. The LD helices are characterized by the formation of a central pore, which can permit passage of cations, a property that is of functional significance in these natural peptides [49].

## 7. CONCLUSIONS

D-amino acids can be effectively used in peptide design since their conformational preferences can facilitate formation of specific folded structures.  $\beta$ -Turn formation is favored in dipeptide sequences of alternating configuration (LD peptides).  $^D$ Pro, a constrained residue, can be used to force the formation of prime turns (type II'/I'), which inturn serves as nuclei for antiparallel  $\beta$ -hairpin formation in designed peptides. Multiple  $^D$ Pro-Xxx segments may be used to promote the formation of multistranded  $\beta$ -sheets. D-amino acids can also be used to mimic helix termination signals like the Schellman motif. Peptides containing building blocks of L- and D- amino acids can be induced to form ambidextrous helices in which right- and left-handed segments are fused. Single D-amino acids and double D-segments may be incorporated into synthetic peptides forming right-handed helical structures. The energy penalties for insertion of a D-residue into a right-handed helix are small and readily compensated by minor structural alterations. Alternating LD sequences provide a special example of the formation of  $\beta$ -helices, which may be viewed as structures generated by winding an extended polypeptide chain around a cylinder. Defining the role of D-amino acids on peptide conformations is of importance in the rational design of analogs of biologically active peptides, which may possess a high degree of resistance to proteolysis.

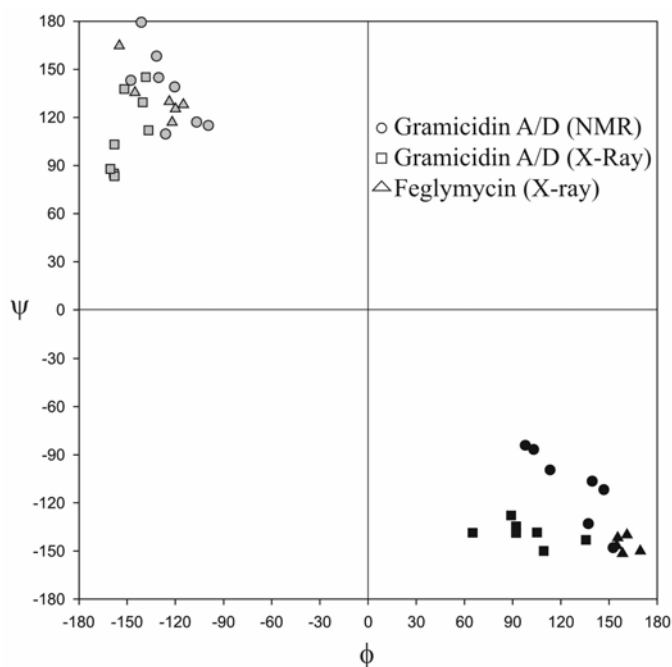


Figure 12. Plot of the observed  $\phi$ - $\psi$  values for the two structures of gramicidin A/D and the crystal structure of feglymycin. It is evident that the dihedral angles of the individual residues populate the extended region of the  $\phi$ - $\psi$  map, although the overall structure formed is helical.

## 8. ACKNOWLEDGMENTS

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