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Retro and retroenantio analogs of cecropin–melittin hybrids

(antibacterial peptides/all-D peptides/peptide topology)

R. B. MERRIFIELD*, PADMAJA JUVVADI*, DAVID ANDREU†, JOSEP UBACH†, ANITA BOMAN‡, AND HANS G. BOMAN‡

*The Rockefeller University, New York, NY 10021; †The University of Barcelona, Martí i Franquès, 1-11, E-08028 Barcelona, Spain; and ‡Stockholm University, S-10691, Stockholm, Sweden

Contributed by R. B. Merrifield, December 22, 1994

ABSTRACT **Hybrid analogs of cecropin A (CA) and melittin (M), which are potent antibacterial peptides, have been synthesized. To understand the structural requirements for this antibacterial activity, we have also synthesized the enantio, retro, and retroenantio isomers of two of the hybrids and their N-terminally acetylated derivatives. All analogs of CA(1–13)M(1–13)-NH_2 were as active as the parent peptide against five test bacterial strains, but one bacterial strain was resistant to the retro and retroenantio derivatives. Similarly, all analogs of CA(1–7)M(2–9)-NH_2 were active against four strains, while two strains were resistant to the retro and retroenantio analogs containing free NH_3^+ end groups, but acetylation restored activity against one of them. From these data it was concluded that chirality of the peptide was not a critical feature, and full activity could be achieved with peptides containing either all L- or all D-amino acids in their respective right-handed or left-handed helical conformations. For most of the bacterial strains, the sequence of these peptides or the direction of the peptide bonds could be critical but not both at the same time. For some strains, both needed to be conserved.**

Antimicrobial peptides have received increasing attention in recent years as their contribution to host defense mechanisms in the animal kingdom is gradually becoming appreciated. Several families of peptide antibiotics from animal sources have now been identified, including the cecropins from insects (1) and pigs (2), the defensins from neutrophils (3), melittin from the honey bee (4), and the magainins from frog skin (5) (for a recent review, see ref. 6). These peptides exhibit broad antibiotic responses against bacteria, protozoa, fungi, and viruses. The mature peptides, derived by the processing of larger precursors, have considerable sequence diversity but share common structural features, including a high content of basic amino acid residues and a global distribution of hydrophobic and hydrophilic residues leading to amphipathic α-helical conformations under hydrophobic conditions. Ion channel formation in artificial membranes has been described for cecropins (7), defensins (8), and magainins (9).

Recent efforts have been directed toward a more detailed understanding of the mechanisms of action of the cecropins and their derivatives through the chemical synthesis of selected analogs. We found that certain 15- to 26-residue hybrids of segments of cecropins combined with segments of other antibacterial peptides, such as melittin, could lead to even more active peptides (10–12) and without introducing the inherent lytic properties of melittin toward eucaryotic cells. It was found that the enantiomers of several of these hybrids, composed of all D-amino acid residues, were fully active against all of our test bacterial strains (13, 14). These results showed that the peptides do not function by chiral interactions with receptors, enzymes, or lipids. In the presence of a hydrophobic lipid bilayer, the peptides can self-aggregate to form pores or channels, which allow the passage of ions under a voltage or concentration gradient. The mechanism leading to the death of a bacterial cell is not yet certain.

We report here our initial results on the identification of the structural features of the cecropin–melittin hybrids that are critical for the development of antibacterial activity.

MATERIALS AND METHODS

Peptide Preparation. All peptides were synthesized by the solid-phase method (15) by using *tert*-butyloxycarbonyl (Boc)-type chemistry as described in detail for cecropin analogs (16). These C-terminal carboxamides were synthesized on *p*-methylbenzhydrylamine resin (17). Thus, both normal and retro peptides and their D-enantiomers were synthesized stepwise in the usual C to N direction using L- or D-amino acids, respectively. N-terminal acetylation, when required, was done with acetic anhydride/*N,N*-diisopropylethylamine (1:1 molar ratio) in *N,N*-dimethylformamide for 20 min at room temperature. HF cleavage and side chain deprotection, followed by reverse-phase liquid chromatography purification, provided peptides of high purity (as determined by HPLC and capillary electrophoresis) that were further characterized by amino acid analysis and mass spectroscopy.

Circular Dichroism Spectroscopy. Spectra were recorded in a Jasco 720 spectropolarimeter. Peptides were dissolved at a concentration of 25 μm in 5 mM sodium phosphate, pH 7.5 ± 0.1, containing hexafluoroisopropanol (HFIP) in various amounts (0–20%, vol/vol). For each HFIP concentration, three scans of data were collected over the 260- to 190-nm interval by using a time constant of 4 s and a 1-nm spectral band. Experimental ellipticity data were converted into molar ellipticities and plotted against wavelength, after noise suppression according to manufacturer protocols. Percent helicity was calculated from a molar ellipticity of $-33{,}300$ m·deg·cm^2·dmol^{-1} at 222 nm for 100% helix.

Antibacterial and Hemolysis Assays. Peptide lytic activity against six bacterial strains and sheep erythrocytes was measured in an inhibition-zone assay on agarose plates (18). For several peptides, independent, duplicate determinations (Stockholm, New York) were performed and found to be in agreement. Lethal concentrations in μM units were calculated from the diameters of inhibition zones as previously described (18). To simplify the presentation of activity data in this paper we have used + to indicate that the activity of the peptide was comparable to the activity of the parent peptide (within a factor of two). Peptides that were inactive or of low activity (<10% of the parent peptide activity) were indicated by −.

RESULTS AND DISCUSSION

The experiments were carried out on the 26-residue cecropin A (CA)–melittin (M) hybrid peptide, CA(1–13)M(1–13)-NH_2,

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Abbreviations: CA, cecropin A; M, melittin; HFIP, hexafluoroisopropanol.

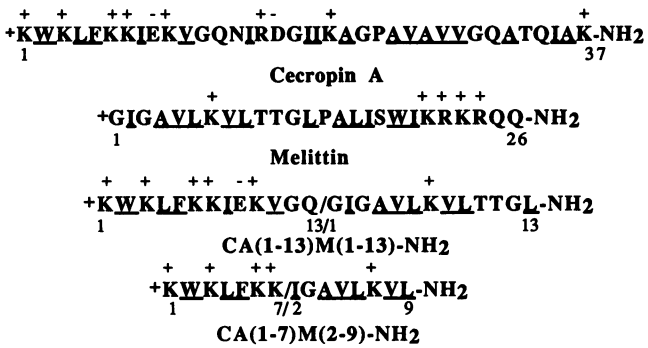


FIG. 1. Sequences of antibacterial peptides.

and the 15-residue hybrid, CA(1–7)M(2–9)-NH_2 (Fig. 1). The parent peptides and their isomers and their N-terminally acetylated derivatives were studied. This allowed the prediction and measurement of the importance of amino acid sequence, direction of the peptide bond, N-terminal charge, chirality of the amino acids, and handedness of the helix. A brief preliminary report on this problem was presented at a recent Ciba Foundation symposium (19).

Structures of the Peptides. Prelog and Gerlach (20) first discussed the topology of cyclic peptides, and the field was developed and reviewed by Shemyakin *et al.* (21). Goodman and Chorev (22) further reviewed the problems of cyclic peptide topology and extended their analysis to linear peptides. Twelve years later Chorev and Goodman (23) reviewed the extensive work on retro and retroinverso linear peptides.

For our purposes we define the cecropin–melittin structures as follows. The parent peptides contain all L-amino acids linked by normal —CO—NH— peptide bonds in the sequence 1, 2, 3,---*n*, numbered from the N terminus (Fig. 2). The enantio peptides contain all D-amino acids linked by normal peptide bonds in the sequence 1, 2, 3,---*n*. They are the mirror images of the parent peptides. The retro peptides contain all L-amino acids linked by normal peptide bonds, but in the reverse sequence *n*,---3, 2, 1. The retroenantio peptides contain all D-amino acids linked by normal peptide bonds in the direction *n*,---3, 2, 1 and are mirror images of the corresponding retro peptides. The L and D acetyl peptides are also mirror images. In these cases it seems reasonable to use the term enantio for the linear, all-D, mirror-image isomers of the parent and retro peptides.

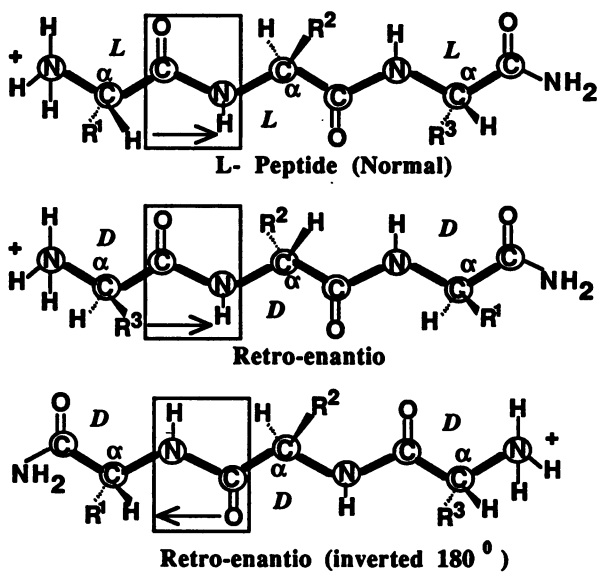


FIG. 2. Linear structures of peptide analogs.

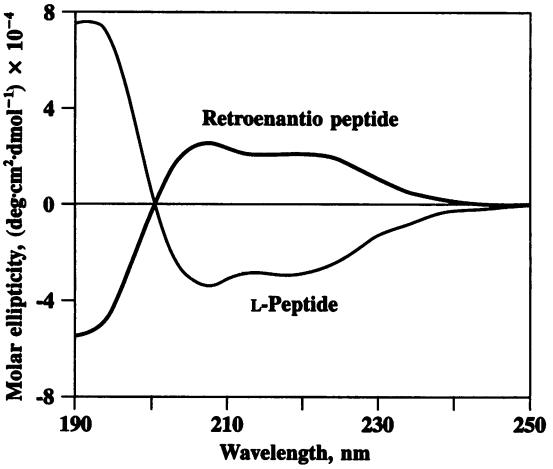


FIG. 3. CD spectra for the parent and retroenantio CA(1–7)M(2–9) -NH_2 peptide in 20% HFIP/5 mM sodium phosphate, pH 7.5.

The retroenantio peptide will, of course, not be a mirror image of the parent peptide. For this reason Goodman and Chorev (22) have preferred to name such an isomer a retroinverso peptide.

The retro and retroenantio peptides can also be viewed from the opposite end by rotating the peptides in the plane 180°, in which case the sequences will read 1, 2, 3,---*n*, but the amide bonds will be —NH—CO— instead of the normal —CO—NH—. If the peptide is a linear random chain or extended conformation, the amino acid side chains of an all-L, retro peptide, when viewed in this reverse direction, will project to the opposite side of the peptide chain and topologically resemble D-amino acid residues. The retroenantio, all-D, peptide side chains, when inverted 180°, will, however, project to the same side as those of the parent all-L peptide (Fig. 2). A retroenantio peptide will therefore have the same sequence and the same side chain topology as the parent, but the amide bonds will be reversed, and the charges on the end groups will be reversed, so it will not be superimposable on the parent peptide. The CD spectra of the parent peptide and retroenantio peptide are not similar, but are related approximately as L- and D-isomers (Fig. 3).

To synthesize a retro peptide, the feature we change experimentally is the sequence. The amide bonds are not constructed backwards but in the normal —CO—NH— direction, and the chirality of the amino acids is the same (L) as in the parent peptide. The ellipticity, therefore, is that of a different sequence and need not be identical with that of the parent peptide—i.e., the tendency for each residue to be in a helix is not necessarily the same in both sequences (Fig. 3). The observed ellipticities at 222 nm in 20% HFIP for seven of the synthetic peptides showed that all the isomers approach a high upper limit of 64–92% helix, while at lower HFIP concentrations the sensitivity to helix formation is much lower and there is much more variation in the degree of helicity of the isomers. The all-L peptides had a negative molar ellipticity at 222 nm and were right-handed helices, whereas the all-D peptides had a positive molar ellipticity and were left-handed helices.

Correlations of Peptide Structural Features with Antibacterial Activity. (*i*) *Random linear structures.* To identify structural features of these cecropin–melittin analogs that are critical for the generation of antibacterial activity, assumptions were first made as to which features were necessary—i.e., must be the same as in the parent peptide, either singly, in pairs, or in triplets—and then the activity was predicted. This gave a + or − pattern for the analogs under study (Table 1). For example, if the only structural requirement for activity is a correct sequence (same as parent), then it is obvious that the enantio peptide will be + because it has the normal sequence,

Table 1. Predicted and observed activities of CA(1–13)M(1–13)-NH_2 and analogs assuming active peptides have a random conformation

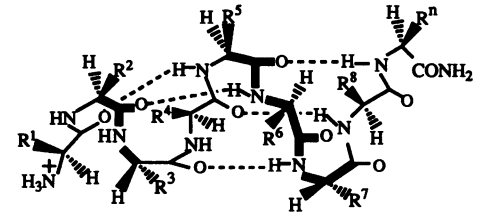
	Normal	Enantio	Retro	Retroenantio	Fit*
	Predicted activity				
Assumed requirement					
1. Sequence	+	+	+	+	
2. Amide direction	+	+	+	+	
3. Chirality	+	+	+	+	
4. Sequence and amide direction	+	+	−	−	
5. Sequence and chirality	+	−	−	+	
6. Amide direction and chirality	+	−	+	−	
7. Sequence, amide direction, and chirality	+	−	−	−	
	Observed activity†				
Organism					
Escherichia coli	+	+	+	+	Sequence or amide direction or chirality
Pseudomonas aeruginosa	+	+	+	+	
Bacillus megaterium	+	+	+	+	
Streptococcus pyogenes	+	+	+	+	
Staphylococcus aureus	+	+	+	+	
Bacillus subtilis	+	+	−	−	Seq. and amide direction

*Fit to predictions for a random conformation.
†The peptides were assayed by the lethal concentration plate method. High activity comparable to the parent peptide is indicated by +, and no or very low activity by −.

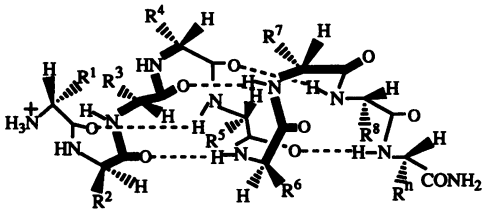
while chirality and amide bond direction are being assumed to be unimportant. The retro and retroenantio peptides have the inverted sequence, but when viewed from the other end the sequence becomes correct and they will both be predicted +, even though the peptide bonds and chirality will again be wrong, because under structural requirement 1, these are being assumed to be unimportant. Similar kinds of arguments can be made for assumed requirements 2 and 3 (Table 1).

Requirements 4–6 assume that two variables must be correct. For number 4 both correct sequence and amide bond direction are considered to be necessary. In this case neither the retro nor the retroenantio will fit, because an adjustment to make the sequence correct will invert the amide bonds and a prediction of − will follow. Similar reasoning for requirements 5 and 6 can be made. Finally, requirement number 7 requires that all three variables be correct at the same time, and only the parent peptide will fit that condition.

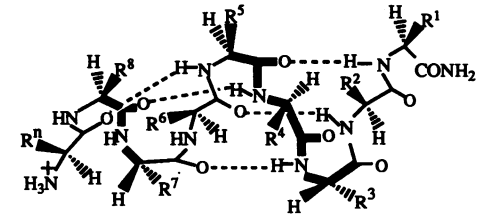
Next, the analogs were tested for activity against six representative bacterial strains. This also gave a + or − pattern that could be compared with the predicted patterns. Data for four analogs of CA(1–13)M(1–13)-NH_2 are shown in Table 1. It was found that five of the organisms responded similarly and that



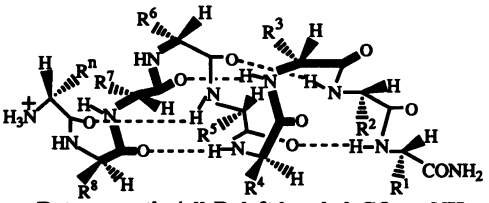
Normal (all-L,right handed, CO→NH; 1,2,3----n)



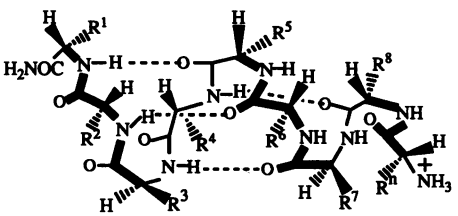
Enantio (all-D, left handed, CO →NH; 1,2,3----n)



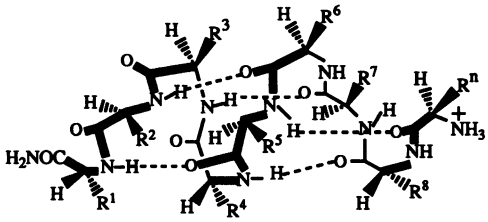
Retro (all-L,right handed, CO →NH; n----3,2,1)



Retro enantio (all-D, left handed, CO →NH; n----3,2,1)



Retro inverted 180° (all-L,right handed, HN←CO; 1,2,3----n)



Retro enantio inverted 180°(all-D, left handed, HN←CO; 1,2,3----n)

FIG. 4. Helical structures of peptide analogs.

Table 2. Predicted and observed activities of CA(1–13)M(1–13)-NH_2 and analogs assuming active peptides are helical

	Normal	Enantio	Retro	Retroenantio	Acetyl normal	Acetyl retro	Acetyl retroenantio	Prediction fit
				Predicted* activity				
Required structure								
1. Sequence	+	+	+	+	+	+	+	
2. Amide bond direction	+	+	+	+	+	+	+	
3. Chirality/handedness	+	−	+	−	+	+	−	
4. Sequence and amide direction	+	+	−	−	+	−	−	
5. Sequence and chirality	+	−	−	−	+	−	−	
6. Amide direction and chirality	+	−	+	−	+	+	−	
7. Sequence, amide direction, and chirality	+	−	−	−	+	−	−	
				Observed activity				
Organism								
E. coli	+	+	+	+	+	+	+	Sequence or amide bond direction
P. aeruginosa	+	+	+	+	+	+	+	Sequence or amide bond direction
Strep. pyogenes	+	+	+	+	+	+	+	Sequence or amide bond direction
Staph. aureus	+	+	+	+	+	+	+	Sequence or amide bond direction
B. subtilis	+	+	−	−	+	−	−	Sequence and amide, direction

*Predictions are the same for CA(1–13)M(1–13)-NH_2 and CA(1–7)M(2–9)-NH_2.

they corresponded to the predicted activities if sequence, amide direction, or chirality was necessary but not if two or three of these features were required at the same time. Therefore, the essential feature could be either sequence, amide direction, or chirality, but from these limited data we cannot decide which of the three is critical. We already had presented evidence (13, 14) that chirality is not critical and need not be the same as the all-L parent peptide, because in ten separate examples the corresponding all-D peptide was as active as the all-L peptide. We believe this simple conclusion is correct, although on the basis of the above discussion, there could be some doubt. Thus, if the D-enantiomer is inverted 180°, then the side-chain topology will resemble that of L-residue side chains, and therefore, chirality is not eliminated as a critical structural feature. However, both the sequence and peptide bond direction will also change, and therefore, to conclude that chirality is important, we must assume that neither sequence nor peptide bond direction is important or needs to be conserved. However, all the data from our laboratory and from others indicate that sequence does play an important role and that not all sequences are active. We conclude that sequence is indeed the important factor and therefore, that chirality and directions of amide bonds need not be critical. Thus, for these five organisms, both —NH—CO— and —CO—NH— bonds in all-D and all-L structures lead to active peptides.

The sixth organism, *B. subtilis*, was more demanding. Its pattern (Table 1) was + + − − and fit only the predicted pattern for a requirement for both correct sequence and correct amide direction (but not chirality). In this case the —NH—CO— bond did not allow for formation of active structures. For all of these peptides the acetylated and nonacetylated versions had equivalent activities, indicating that the N-terminal amine could be charged or not charged and retain activity.

(ii) Helical structures. In contrast to the random configuration, if we consider these antibacterial peptides to be α-helical in their active conformations, different predictions will result (Fig. 4). This assumption that the active cecropins and hybrids are amphipathic helices is well supported by data from several laboratories (16, 24–26). The parent peptide will be all-L and a right-handed helix. The enantiomer will be all-D and a left-handed helix. They will be mirror images. This is supported by the CD spectra, which are exact mirror images (13, 14). The retro peptide will contain all L-amino acids in normal peptide bonds and a right-handed helix but of opposite sequence (*n*,---3,2,1). The retroenantio peptide will contain all D-amino acids in normal peptide bonds but of opposite sequence (*n*,---3,2,1) and in a left-handed helix. It is a mirror image of the retro peptide (data not shown).

When the retroenantio peptide is viewed from the opposite end, it will have the same sequence as the parent peptide, and the side chains will project from the same side as in the L-peptide, but the amide bonds will be reversed and the end group charges will be reversed. However, the conformation of the helix will remain left-handed in contrast to the right-handed helix of the parent peptide. As shown in Table 2, it is expected that the helical conformation will not allow the chirality or helical handedness of the enantio, retroenantio, or acetyl retroenantio peptides to be the same as in the normal parent peptide, even if sequence or amide bonds are inverted. Therefore, we predict that these analogs will be inactive if chirality or handedness is an essential structural feature. Since all of the analogs of CA(1–13)M(1–13)-NH_2 were fully active against four of the test organisms, *E. coli*, *P. aeruginosa*, *Strep. pyogenes*, and *Staph. aureus* (Table 2), the fit with prediction is with sequence only or with amide direction only, and chirality is firmly eliminated as an essential feature. With the helical model, *B. subtilis* still fits the prediction that both sequence and amide bond direction must be the same as the parent peptide. For this organism also, chirality is eliminated as an essential feature.

The data in Table 3 are the observed activities of CA(1–7)-M(2–9)-NH_2 and analogs. For this shorter, 15-residue hybrid, all isomers were active against four of the test organisms, *E. coli*, *B. subtilis*, *B. megaterium*, and *Staph. aureus*. As with the longer, 26-residue peptide, these data match the predictions for either sequence, amide direction, or chirality alone if the peptide is a random coil, but if it is a helix, then they match sequence or amide alone but not chirality as a necessary requirement. The data for *P. aeruginosa* agree with the prediction that both sequence and amide direction must be correct.

A different result was obtained with the short peptides in the case of *Staph. aureus*. The retro and retroenantio analogs were both inactive (only 0.5–1% active), but the corresponding acetylated derivatives were active. This indicates that the location of the -NH_3^+ charge can have a profound effect. In these two isomers the -NH_3^+ is on residue *n* (Leu) rather than residue 1 (Lys). The latter is in the basic end of the molecule, whereas the former is in the more hydrophobic, less charged end. Thus, the important feature for activity is either sequence or amide direction, provided an -NH_3^+ is not present on residue *n*. Similar results were not found in the 26-residue series,

Table 3. Observed activity of CA(1–7)M(2–9)-NH_2 and analogs

Organism	Normal	Enantio	Retro	Retroenantio	Acetyl retro	Acetyl retroenantio	Prediction fit: Random	Prediction fit: Helix
E. coli	+	+	+	+	+	+	Sequence or amide bond direction or chirality	Sequence or amide bond direction
B. subtilis	+	+	+	+	+	+		
B. megaterium	+	+	+	+	+	+		
Strep. pyogenes	+	+	+	+	+	+		
Staph. aureus	+	+	−	−	+	+	Sequence or amide or chirality but no + on residue *n*	Sequence or amide direction but no + on residue *n*
P. aeruginosa	+	+	−	−	−	−	Sequence and amide	Sequence and amide

suggesting that the + charge alteration has a much larger effect on the conformation or aggregation of the small peptides.

Our data show that the most important feature for activity in this class of linear antibacterial peptides is the order of the side chains on a helical molecule and that direction of amide bonds and chirality can be reversed without affecting the activity against most of the test bacteria. For some organisms, both sequence and peptide direction must be conserved.

There remain varying views of the mechanism of killing and lysis of susceptible bacteria by these peptides. The lethal concentrations of many peptides are roughly equivalent to the concentrations needed to produce a surface monolayer (29), and detergent effects have been suggested as the mechanism for membrane disruptions. It is clear from earlier work that such amphipathic helical peptides can give rise to ion channel (pore) formation in artificial membranes (7–9). In addition, solid-state NMR (27) and fluorescence spectroscopy (28) have shown that certain helix-forming peptides can insert into lipid bilayers either primarily perpendicular or primarily parallel to the plane. Extrapolation of such data to the effects on liposomes, or mitochondrial (30) or bacterial membranes is difficult, but eventually the exact mechanism or mechanisms of action must accommodate all the data from these varied approaches, including the new results with the retro and retroenantio peptides.

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