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Synthesis and characterization of 1-^{13}C-D · Leu$^{12, 14}$ gramicidin A

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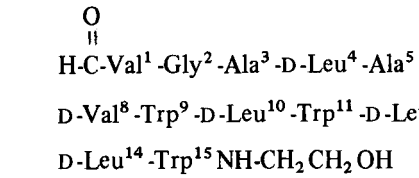
The ^{13}C-D-Leu^{12,14} gramicidin A was synthesized by the solid phase method incorporating ^{13}C-D-leucine in positions 12 and 14 with about 25 and 50% enrichment, respectively. The pentadecapeptide was removed from the resin by ethanolamine treatment, with the *N*-protecting group (Boc) still on. After removal of the protecting group, the peptide was formylated and purified by preparative t.l.c. to obtain ^{13}C-D-Leu^{12,14} gramicidin A in a very pure state in an overall yield of about 12.5%. The peptide was then thoroughly characterized by HPLC which gave one single peak with the same retention time as that of Val^1-gramicidin A of the natural gramicidin mixture. The CD spectra of the synthetic and the HPLC purified natural Val^1-GA were obtained and found to be identical, indicating the optical purity of the sample. The synthetic GA was characterized by ^{13}C n.m.r. spectrum and compared with that of natural GA. Single channel conductance parameters of the synthetic GA were determined and found to be indistinguishable from those of natural Val^1-GA in lipid bilayer membranes and the mean channel lifetime was found to be as reported earlier by others.

Key words: carbon-13 magnetic resonance; circular dichroism; conductance properties; gramicidin A; HPLC; L,D-Leu resolution; single channel currents and synthesis.

Naturally occurring gramicidin isolated from a strain of *Bacillus brevis* (1) has been shown to occur as a mixture of gramicidin A, B and C (2) in the ratio of 7:1:2 (3). Sarges & Witkop (4) have determined the primary sequence of the gramicidin A to be:



Gramicidin B has Phe in place of Trp^{11} and there is a Tyr in place of Trp^{11} in gramicidin C (5).

There is great interest in this peptide since it forms channels in biological membranes resembling Na channels of the nerve membrane in some of its electrical properties (6, 7). Gramicidin A forms transmembrane channels with monovalent cation selectivity (8–11). The structure of the gramicidin channel was proposed by Urry (12, 13) and in this model two molecules of gramicidin come together at their *N*-termini (formyl end to formyl end) to form the channel with the length of 26 Å and a pore diameter of 4 Å. The β-helical structure of the dimeric channel is stabilized by six intermolecular hydrogen bonds (with the terminal formyl groups contributing to two of the hydrogen bonds) and 22 intra-

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molecular hydrogen bonds. The studies on the channel formation kinetics of gramicidin A (14), the fluorescence studies (15) and the conductance and concentration studies of Apell *et al.* (16) have provided evidence for this model. The same conclusion was also derived by Weinstein *et al.* (17, 18) from their ^{19}F and ^{13}C n.m.r. studies on gramicidin analogs, which excluded the alternate double stranded helical model originally proposed by Veatch *et al.* (19, 20). The presence of two binding sites in K^+ and Cs^+ complexes of gramicidin was demonstrated by X-ray diffraction studies (21) and Urry *et al.* (22) have observed two binding sites in lysolecithin incorporated malonyl-bis-desformyl gramicidin channels using sodium-23 n.m.r. spectroscopic studies. Still the exact location of these ions, i.e. the ion binding sites inside the channel, is not known when the channel is incorporated into phospholipid structures. Even in the crystal structure the ion locations are not positioned, as yet, with respect to the ends of the channel with the observation being that the two sites are separated by 21 and 5 Å along the end-to-end aligned channels.

The transmembrane channel of gramicidin has all the hydrophobic side chains on the exterior and the peptide carbonyls lining the inside wall of the channel. As the ion traverses the channel, it interacts with the peptide carbonyls. So one approach to pinpointing the exact binding sites of the gramicidin channel is to label, individually, the peptide carbonyls with C-13 enriched amino acids and to study the cation interaction after incorporating the C-13 enriched gramicidin analog into phospholipid structures. Without enrichment, it is not possible to observe and assign the carbonyl resonances in the natural abundance (~ 1.1% ^{13}C) spectrum of the gramicidin in the lipid structures due in part to the immobilization of peptide inside the lipid core of the phospholipid and also due to its overall low concentration in the solution (23, 24). For this purpose we are now synthesizing several gramicidin analogs incorporating C-13 enriched amino acids at various positions in the gramicidin. Here we report the synthesis by the solid phase method (25), purification and characterization of one such gramicidin A analog with 1-^{13}C-D-leucine in position 12 (~ 25% enrichment) and in position 14 (~ 50% enrichment) which we refer to as ^{13}C-D-Leu^{12,14} gramicidin A. The synthesis of gramicidin has been carried out by the classical solution method (4, 26) and also by the solid phase method (27, 28). The presence of four tryptophan residues is of great concern since the indole ring of tryptophan would undergo oxidative degradation under acidic conditions used for the removal of *N*-protecting Boc-group (29, 30). The second major concern is the purification of the finished peptide. It is well known that gramicidin molecules associate in solution and this property allows the short chain peptides and the acetylated peptide impurities to associate with the required gramicidin analog, thus making purification difficult by the usual column chromatographic methods.

MATERIALS AND METHODS

Ascending thin-layer chromatography (t.l.c.) was carried out on Whatman silica gel plates (80 Å); ninhydrin and chlorine-*o*-tolidine/KI sprays were used to identify the spots on t.l.c. plates. All the amino acids used are of the L-configuration unless otherwise indicated. Boc-amino acids were either prepared in our laboratory or obtained from Bachem, Inc., Torrance, California and Peninsula Laboratories, Inc., San Carlos, California. 1-^{13}C-DL-leucine (92% enrichment) was obtained from KOR Isotopes, Cambridge, Massachusetts. Ion exchange resins were obtained from Bio-Rad, Richmond, California. Amino acid analyses were performed on a Beckman 119H amino acid analyzer using the ninhydrin method, [with pH 3.25 (0.2 M Na^+) buffer (100 min), pH 4.25 (0.2 M Na^+) buffer (40 min) and pH 6.5 (1.1 M Na^+) buffer (100 min)]. Samples were hydrolyzed using 1) 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole for 22 h at 115°, and 2) 6 N HCl for 24 h at 115° in evacuated sealed tubes. HPLC was run on a Beckman Model 332 gradient liquid chromatograph using an Ultrasphere ODS column (25 cm x 4.6 mm) at room temperature with u.v. monitoring at 220 nm. CD spectra were recorded with a Cary 60 spectropolari-

meter equipped with a model 6001 circular dichroism attachment modified for high frequency modulation. The carbon-13 magnetic resonance spectra were obtained on a JEOL-FX-100 spectrometer equipped with a 10-mm probe operating with a deuterium lock and proton noise decoupling. The spectrometer was operated in the pulse-Fourier transform mode by a Texas Instruments 980B computer (48K memory) with foreground-background data acquisition and analysis capability. The probe temperature was maintained at $30 \pm 2°$. Samples of natural gramicidin at 0.027 M (ICN Pharmaceuticals, Inc., Cleveland, Ohio) and the synthetic gramicidin A at 0.009 M were dissolved in dimethyl-d_6-sulfoxide (99.5% D, Merck, Sharp and Dohme, Montreal, Canada).

Single channel conductance measurements

The single channel parameters of 1-^{13}C-D-Leu^{12,14} gramicidin A were compared with those of purified natural Val^1-gramicidin A. Val^1-gramicidin A was isolated from commercial gramicidin (ICN Pharmaceuticals, Inc.) using HPLC. Both compounds were stored dry until the day of the experiment at which point they were dissolved in methanol. Lipid bilayer membranes were formed from dispersions of monoolein (50 mg, Nu-Check) in hexadecane (1 ml, Aldrich gold label) on apertures of 65 or 90 μm diameter. The aperture was fashioned from polyethylene pipettes by melting and shaving their tip. The measurement chamber was constructed by press-fitting the pipette into a teflon block containing a 3-ml reservoir. The reservoir and the pipette were filled with unbuffered (pH ~ 5.6) aqueous 1 M KCl solution. The potential between silver-silver chloride wire electrodes in the reservoir and pipette was measured before and after each experiment. With the reservoir electrode held at + 100 or − 100 mV and the pipette electrode at virtual ground, the current passing into a current-to-voltage converter (10^9 Ohm feedback resistor) was monitored as a membrane formed. When the membrane was verified to be noise-free, 2–4 μl of gramicidin solution (10^{-5} mg natural gramicidin A or 10^{-6} mg synthetic gramicidin A per ml methanol) was added to the reservoir until moderately frequent current transitions were observed. The output of the current-to-voltage converter was amplified 100×, filtered at 100 Hz, sampled at 300 points per second, and stored digitally. The currents were subsequently analyzed semi-automatically with the assistance of a PDP 11/70 computer. The histograms were formed by sorting the transitions into bins according to their magnitude and dividing the number of transitions in each bin by the total number of transitions observed in the experiment. The abscissa is labeled according to the conductance represented by the center of the bin, the bin width being 0.0244 pS. The ordinate shows the fraction of transitions in a given bin. The root mean square baseline noise was 0.07 pS.

Synthesis

N-Acetyl-1-^{13}C-DL-leucine (I). A solution of 2.0 g (15.24 mmol) of 1-^{13}C-DL-leucine in gl. HOAc (20 ml) was brought to boil and acetic anhydride (3.16 ml; 33.49 mmol) added drop by drop. The reaction mixture was refluxed for 10 min more and cooled for 2 h. Solvent was removed under reduced pressure, water added and once again evaporated. The residue was taken in warm acetone (50 ml), treated with norite, filtered and concentrated to a small volume and left at room temperature overnight. The crystals which appeared were filtered, washed with acetone and ether, and then dried. A second and third crop of crystals were collected, 2.09 g (yield: 79.2%) and t.l.c. gave a single spot in EtOAc:HOAc:EtOH (9:1:1) and $CHCl_3$:CH_3OH:HOAc (95:5:3) solvent systems.

Acetyl-1-^{13}C-D-leucine (II). I (2.0 g, 11.54 mmol) was suspended in water (92.5 ml) and the pH brought to 7.2 by the addition of conc. ammonia solution. Hog kidney acylase I (20 mg, Nutritional Biochemical Corp.) was added and incubated at 37° for 24 h. Only a small amount of the formation of L-leucine could be noticed on t.l.c. More of the enzyme (200 mg) was added and incubation continued for one more day. The pH of the solution was brought to 2 using 1 N HCl and extracted several times with ethyl acetate. The combined

EtOAc extracts were concentrated to obtain 0.85 g acetyl-1-^{13}C-D-leucine.

1-^{13}C-D-leucine (III). A solution of II (0.85 g, 4.9 mmol) in 2 N HCl (24.5 ml) was refluxed for 2 h and solvent removed under reduced pressure. The residue was taken in a minimum amount of water (10–15 ml), and pH adjusted to 5 with pyridine. The precipitate obtained was redissolved by warming and 30 ml hot ethanol added and left in the refrigerator overnight. The crystals of 1-^{13}C-D-leucine were filtered, washed with a little water, with ethanol and dried. The filtrate was concentrated *in vacuo*; the residue was taken in 5 ml water, the pH adjusted to 6 and left in the refrigerator for several hours to obtain a second crop giving a total of 530 mg of the product (yield: 82.55%). The pH of the solution of 1-^{13}C-D-leucine (0.52 g) in 15 ml water was adjusted to 7.2 by adding 0.2 M tris-buffer and incubated at 37° with 5 mg of L-amino acid oxidase (Millipore Corporation, Freehold, New Jersey). After 24 h, an additional 5 mg of the enzyme was added, and the incubation was continued for one more day. The pH of the solution was adjusted to 5 and dialyzed against water (25 ml), changing the water every 2 h. The combined aqueous portion (125 ml) was treated with norite, filtered and concentrated. The residue was taken up in 15 ml water; 15 ml ethanol were added, and the sample was left in the refrigerator. The crystals obtained were filtered, washed with ethanol and dried. A second crop of crystals was obtained from the mother liquor yielding a total of 432 mg optically pure 1-^{13}C-D-leucine as verified by CD spectrum (see Fig. 1).

Boc-1-^{13}C-D-leucine (IV). To ^{13}C-D-leucine (227.96 mg; 1.737 mmol), a solution of 69.5 mg NaOH in 0.17 ml water was added followed by 0.35 ml *tert.*-butanol and mixed thoroughly. Di-*tert.*-butyl-dicarbonate (471 mg) was added slowly and mixed for a short time. More *tert.*-butanol (0.35 ml) was added and stirred overnight. Water (0.9 ml) was added, stirred and extracted with ether. The aqueous portion was cooled, acidified to pH 2 with 1 N HCl and extracted with EtOAc (3 x). The combined EtOAc extracts were washed once with satd. NaCl, dried over anhyd. $MgSO_4$, and solvent was removed under reduced pressure to obtain 339 mg of the product (yield: 78.33% considering Boc-^{13}C-D-Leu·H_2O as the product). T.l.c. in CMA (95.5:3) gave a single spot (see CD spectra, Fig. 1).

Boc-Val-Gly-Ala-D-Leu-Ala-D-Val-Val-D-Val-Trp-D-Leu-Trp-^{13}C-D-Leu-Trp-^{13}C-D-Leu-Trp-$NHCH_2CH_2OH$ (V). The synthesis was carried out manually using Boc-Trp-resin (3.30 g) containing a total of 1 mmol of Boc-Trp-OH esterified to 1% cross-linked resin (Peninsula Laboratories, Inc., San Carlos, CA). The following steps were performed to incorporate each amino acid: 1) washings with CH_2Cl_2 (3 x) (10 ml solvent/g resin; 2) 33% TFA/CH_2Cl_2

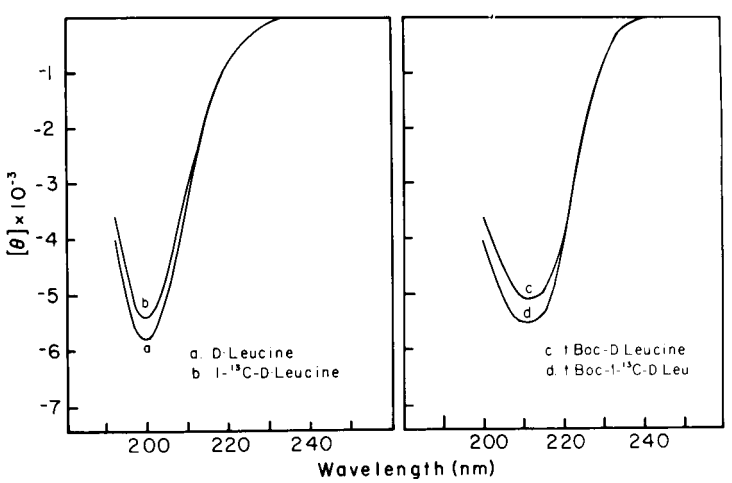

FIGURE 1

CD spectra of a) D-leucine (Sigma) and b) 1-^{13}C-D-leucine in water and c) Boc-D-leucine (Peninsula Labs) and d) Boc-1-^{13}C-D-leucine in methanol.

containing 2% 1,2-ethanedithiol, 5 min and 25 min; 3) washings with CH_2Cl_2 (6×); 4) 5% diisopropylethyl amine in CH_2Cl_2, 2× 5 min; 5) washings with CH_2Cl_2 (6×); 6) 2.5 fold excess Boc-amino acid and mix 2 min; 7) 2.5 fold excess DCC and reacted for 4 h; 8) washings with CH_2Cl_2 (3×); 9) washings with EtOH (3×); 10) washings with CH_2Cl_2 (6×); 11) coupling with 1.5 fold excess of Boc-amino acid and DCC in 50% DMF/CH_2Cl_2 to react overnight; 12) washings with CH_2Cl_2 (3×); 13) washings with EtOH (3×); 14) check by Kaiser test; 15) washings with CH_2Cl_2 (5×); 16) acetylation with 10 equiv. acetic anhydride and 5 equiv. Et_3N in CH_2Cl_2-60 min; 17) washings with CH_2Cl_2 (6×) and continue with step 2 for the next coupling. Boc-D-Leu and Boc-Trp were first wetted with a few drops of DMF and then diluted with CH_2Cl_2 for the coupling reaction. The Boc-$Val^1 \cdots Trp^{15}$-resin (5.87 g) was suspended in a mixture of methanol (100 ml) and distilled ethanolamine (50 ml) and stirred at 60° for 40 h in a sealed flask. The resin was filtered, washed with methanol and dried. Methanol was removed from the filtrate; and the peptide was precipitated by the addition of water; filtered, washed with water and dried (1.42 g). A second crop of 0.40 g of the peptide was obtained by a further treatment of the resin with ethanolamine as above.

Desformyl-^{13}C-D-Leu^{12,14} gramicidin A (VI). A solution of V (0.70 g) in 33% TFA/$CHCl_3$ containing 5% 1,2-ethanedithiol (20 ml) was stirred for 30 min under a nitrogen atmosphere. Solvent was removed under reduced pressure and dried over P_2O_5 and NaOH in a vacuum desiccator. The TFA salt of the peptide was taken in methanol (50 ml) and passed through a column of AG 50W-X2 (H^+ form) resin (40 ml) equilibrated with methanol and eluted with methanol (600 ml) in a cold room. Elution was then continued with methanolic ammonia (1600 ml MeOH + 320 ml conc. ammonium hydroxide) to obtain 546 mg crude desformyl ^{13}C-D-$Leu^{12,14}$ gramicidin.

^{13}C-D-Leu^{12,14}-gramicidin (VII). VI (546 mg) was taken in 95–97% formic acid (7.3 ml) and cooled with ice-water. Acetic anhydride (2.18 ml) was added drop by drop while stirring, over a period of 15 min and stirring was continued for 30 min at 0° and 4 h at room temperature. Solvent was removed under reduced pressure and dried. The residue was taken in MeOH (11 ml) and treated with 1 N NaOH (2.2 ml) for 1 h. The reaction mixture was passed through a column of AG 50W-X2 (H^+ form) resin (40 ml) and eluted with methanol to obtain 485 mg of the formylated product. The peptide was purified by preparative t.l.c. using the CMA (85:15:3) solvent system for developing the plates. The t.l.c. band having the same R_f value as the natural gramicidin A was separated and the peptide was extracted with 15% MeOH/acetone. The solvent was removed under reduced pressure, the residue was taken in a small amount of MeOH and the peptide precipitated by the addition of water. The precipitate obtained was filtered, washed with water and dried to obtain 89.90 mg of pure ^{13}C-D-$Leu^{12,14}$ gramicidin A (overall yield of 12.4%). T.l.c. indicated the same number of multiple spots as the natural gramicidin in the solvent system pyridine-butanone-2 (3:7) and gave a single spot in CMA (85:15:3) with an R_f value 0.63. A small fraction of the sample was further purified by HPLC for black lipid membrane studies.

RESULTS AND DISCUSSION

Since 1-^{13}C-D-leucine was not available commercially, the amino acid was obtained by the enzymatic resolution of 1-^{13}C-L,D-leucine (31). Boc-^{13}C-D-leucine was then prepared using di-*tert.*-butyl carbonate (32). The purity of the product was checked by comparing the CD spectra with standard samples (Fig. 1) and the ellipticity values are presented in Table 1. Within the experimental error, the results indicate that the resolution was achieved satisfactorily. The scheme for the synthesis of gramicidin is outlined in the experimental section. The removal of the Boc group was carried out using 33% TFA/CH_2Cl_2 containing 2% 1,2-ethanedithiol to suppress the oxidative destruction of tryptophan (33). While incorporating ^{13}C-D-leucine in position 14 (~ 50% enrichment) and position 12 (~ 25%

TABLE 1

Ellipticity values of amino acids and peptides

Sample	γmax (nm)	[θ]
D-Leucine (Sigma)	200	5.7×10^{-3}
^{13}C-D-leucine	200	5.4×10^{-3}
Boc-D-leucine (Peninsula Labs)	212	5.0×10^{-3}
Boc-^{13}C-D-leucine	212	5.6×10^{-3}
Val^{1}-GA (HPLC pure)	232	1.92×10^{-3}
^{13}C-D-Leu^{12,14}-GA	215	2.209×10^{-3}

enrichment), only 1 equiv. of Boc AA was used for the first coupling and two successive couplings were carried out with 0.5 equiv. each time. The completeness of the reaction was checked by the Kaiser test (34). After removing the peptide from the resin and deblocking the *N*-terminal Boc group the desformyl gramicidin was passed through a cation exchange column (AG 50W-X2 H^+ form) to remove most of the acetylated products and unreacted peptide by eluting with methanol and the required product was obtained by eluting with 2 N methanolic ammonia solution. The same degree of purification was not achieved when AG 50W-X8 cation exchange resin was used. Two different batches of X8 resins were tried without success.

Purification of the formylated peptide using an LH-20 column was not successful, since the required product always eluted in association with impurities. A very good purification of the peptide was achieved by preparative thin-layer chromatography using the chloroform:methanol:acetic acid (CMA) (85:15:3) solvent system. Gramicidin A was eluted from the silica gel using 15% methanol in acetone.

The purity of the peptide was checked and compared with natural gramicidin. The synthetic material gave the same characteristic multiple spots as the natural peptide in the pyridine:butanone-2 (3:7) solvent system and gave a single spot in the above CMA solvent system.

The results of amino acid analyses are presented in Table 2. When the hydrolysis was carried out using 4 N methanesulfonic acid (35), valine values were always low, even though a complete recovery of tryptophan was obtained. The reason may be due to incomplete hydrolysis of the H-C(=O)-Val-bond and/or the D-Val^{6}-Val^{7}-D-Val^{8} peptide sequence. A full recovery of valine was found when *N*-formyl-L-valine was hydrolyzed by the same procedure. Hydrolysis of gramicidin samples with 6 N HCl resulted in full recovery of valine. These results indicate that D-Val-Val-D-Val peptide sequence is resistant to 4 N methanesulfonic acid hydrolysis under the experimental conditions used.

HPLC data on the synthetic and natural gramicidin samples is presented in Fig. 2. The retention time for the synthetic material was exactly the same as the Val^{1}-gramicidin fraction in the natural material, and which was also isolated by preparative HPLC of gramicidin mixture. The solvent system, 15%

TABLE 2

Amino acid analysis of natural gramicidin mixture and synthetic ^{13}C-D-Leu^{12,14}-GA

Amino acid	4 N methanesulfonic acid hydrolysis, 115°, 22 h		6 N HCl hydrolysis, 110°, 24 h		
	Natural GA	Synthetic GA	Natural GA	Synthetic GA	Theory
Glycine	1.1	0.91	1.02	1.1	1.0
Alanine	2.1	2.1	1.99	2.0	2.0
Valine	2.83[a]	3.22[a]	3.83	3.76	4.0
Leucine	4.0	4.0	4.0	4.0	4.0
Ethanolamine	1.1	1.08	0.93	0.97	1.0
Ammonia	0.16	0.2	0.25	0.26	
Tryptophan	3.9	3.94	3.52	3.69	4.0

[a] These low values are due to incomplete hydrolysis of the peptide (see Discussion).

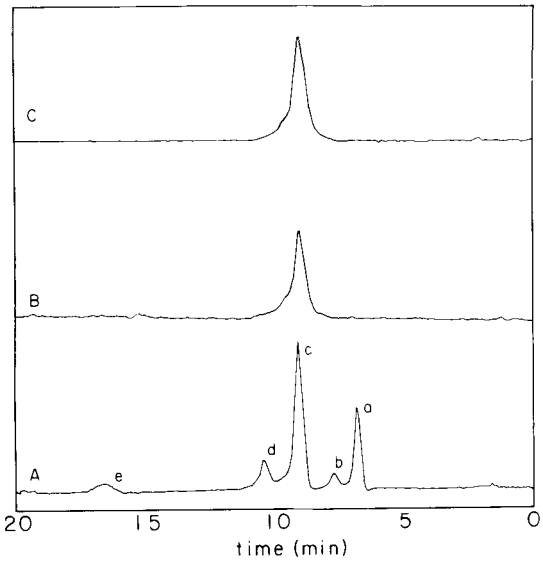

FIGURE 2

HPLC of A) natural gramicidin mxiture B) natural Val^1-gramicidin A isolated by preparative HPLC and C) synthetic ^{13}C-D-Leu^{12,14}-GA on Altex Ultrasphere ODS analytical column using 15% water in methanol solvent system at room temperature.

water in MeOH gave the optimum resolution on the Altex Ultrasphere ODS analytical column (4.6 mm x 25 cm) at room temperature. The peptide is so sensitive to the solvent system that either a decrease or an increase of water content by 1% did not give the same resolution for the natural gramicidin. When the peptides were isolated from HPLC column, there was always contamination of silica gel eluted from the column and further purification (e.g. passing through ion-exchange column or gel filtration over an LH-20 column) has to be carried out to get rid of silica gel. The concentration of Val^1-gramicidin was determined by u.v. absorbance with (ϵ) at 282 nm of 22 500 in methanol for concentrations near 1 mM. The presence of silica gel is apparent from the area of the peptide peak obtained by running the samples again on HPLC, and also from the amounts of synthetic and natural Val^1-gramicidin A samples that have to be used for the black lipid membrane studies.

The CD spectra of Val^1-gramicidin A isolated by HPLC and the synthetic gramicidin are presented in Fig. 3 and the ellipticity values are presented in Table 1. Both the samples gave identical spectra with the superimposed runs being indistinguishable.

The carbon-13 magnetic resonance spectra in dimethyl-d_6-sulfoxide of natural gramicidin A′ and the synthetic ^{13}C-D-Leu^{12,14} gramicidin A are presented in Fig. 4. The assignments for the natural material have been reported previously (36) except for the Gly^2*C*=0 which has been assigned in this laboratory using Edman degradation products of gramicidin (unpublished results). It may be observed that the spectrum of the synthetic product compares very well with that of the natural material. The extra resonances which occur in the aromatic tryptophan indole region of natural gramicidin A′ are from the aromatic side chains of the B and C analogs which contain phenylalanine and tyrosine in position eleven, respectively. The intense signal at 171.6 p.p.m. in the carbonyl region of the synthetic material is, of course, due to the 1-^{13}C enriched residues of D-Leu^{12,14}.

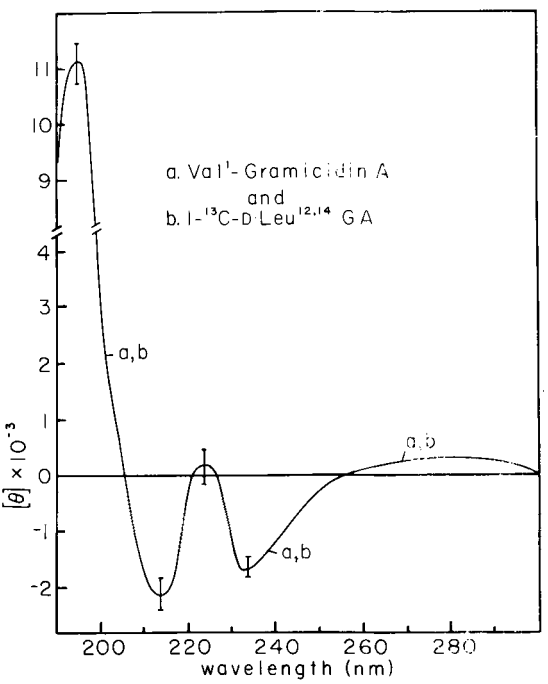

FIGURE 3

CD spectra of a) natural Val^1-gramicidin A and b) ^{13}C-D-Leu^{12,14}-GA.

Single channel conductance

Fig. 5 compares typical single channel records of (a) natural gramicidin A, and (b) synthetic 1-^{13}C-D·Leu^{12,14} gramicidin A. It is seen from these records that the two gramicidins form channels that are indistinguishable. Careful examination of the full current records revealed that all but one or two of the 490 1-^{13}C-D·Leu^{12,14} gramicidin channels observed had the same characteristics as natural gramicidin channels, namely (a) they started and stopped conducting abruptly (in less than 3 ms), and (b) in most cases conducted at a constant level throughout their lifetime.

Fig. 6 compares the single channel conductance histogram of (a) gramicidin A with (b) 1-^{13}C-D·Leu^{12,14} gramicidin A. The experiments contained 1176 and 980 transitions, respectively. Most of the transitions (50% and 70%, respectively, for synthetic and natural gramicidin A) fell within a sharp, single main peak. Judging by visual criteria, the shape of each peak could be fit well (i.e. snugly) by a normal curve, giving a mean transition amplitude of 45.2 pS (S.D. = 0.4 pS) for synthetic gramicidin A and 44.9 pS (S.D. = 0.5 pS) for natural gramicidin A. The difference in conductance is within tolerances determined by fluctuations in room temperature, 23° ± 1°, at which these experiments were done. In other experiments done under similar

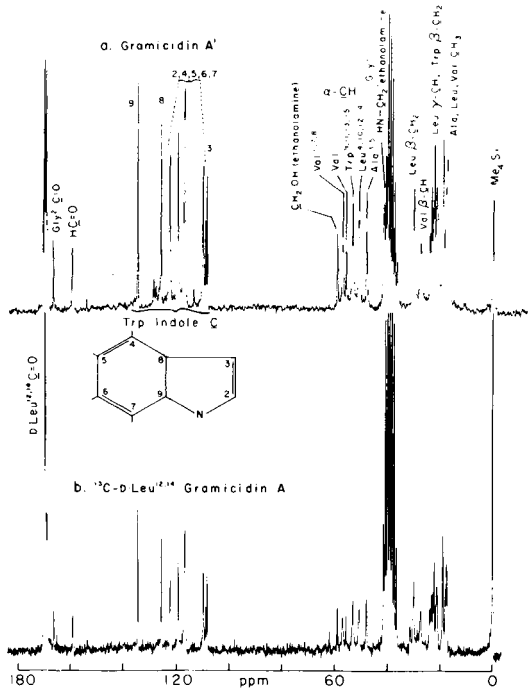

FIGURE 4

Carbon-13 magnetic resonance spectra at 25 MHz of a) natural gramicidin mixture (gramicidin A') and b) synthetic ^{13}C-D-Leu^{12,14} gramicidin A in dimethyl-d_6-sulfoxide at 30°.

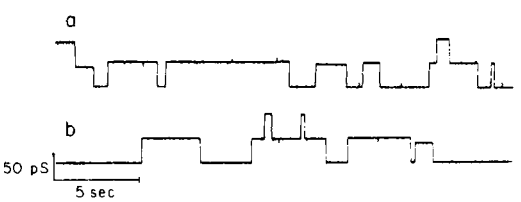

FIGURE 5

Single channel currents for a) HPLC purified (natural) gramicidin A and b) HPLC purified 1-^{13}C-D·Leu^{12,14} gramicidin A in GMO/hexadecane (50 mg/ml) bilayers at 23 ± 1° using 1 M KCl, 103 mV.

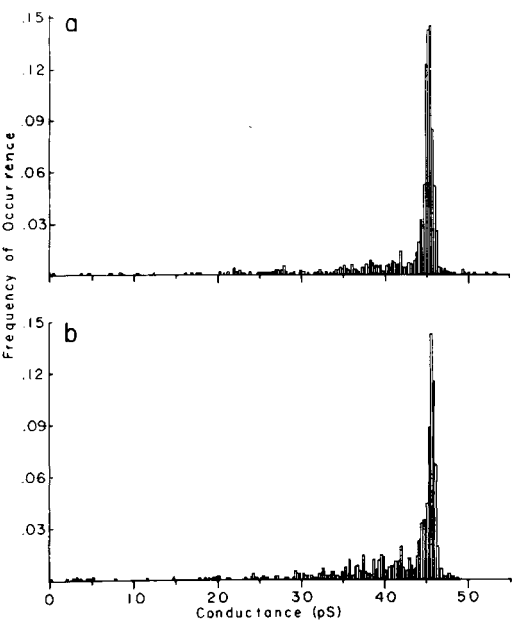

FIGURE 6

Transition amplitude histograms for a) HPLC purified ICN (natural) gramicidin A and b) HPLC purified 1-^{13}C-D·Leu^{12,14} gramicidin A in GMO/hexadecane (50 mg/ml) bilayers at 23 ± 1° using 1 M KCl. The transmembrane potential was 103 mV. RMS baseline noise was 2–3 histogram bins.

conditions but using gramicidin A which had been purified by HPLC previously, the main peak contained variable fractions of the total number of transitions, ranging from 50% to 80%. Thus the amplitude histograms do not appear to differ substantially in either the fraction of channels contained in, or the position of, the main peak.

The mean single channel lifetime for channels whose conductance fell within the main peak group was 3.0 s (n = 255, S.D. = 4.0) for 1-^{13}C-D·Leu^{12,14} gramicidin A, and 4.2 s (n = 316, S.D. = 4.6) for natural gramicidin A. In a separate experiment, again using previously purified natural gramicidin, a mean channel lifetime of 3.0 s (n = 200) was obtained under similar conditions and others have published similar values (9, 37, 38). The difference between these lifetimes falls within the usual range of reproducibility. We conclude, in summary, that the synthetic and natural gramicidin A have identical electrical properties.

In summary, 1-^{13}C-DL-leucine was enzymatically resolved and the required ^{13}C-D-leucine was converted to Boc-derivative using di-*tert.*-butyl-dicarbonate. The pentadecapeptide, was synthesized by the solid phase method with ^{13}C-enriched D-leucine in positions 12 and 14 with about 25 and 50% enrichment respectively. The *N*-protected (Boc) peptide was removed from the resin by ethanolamine treatment. After removing the Boc-group, the peptide was passed through an ion-exchange column to remove the acetylated peptide impurities while the desformyl GA was eluted with methanolic ammonia. After formylation, the ^{13}C-D-Leu^{12,14} GA was obtained in a very pure state by preparative t.l.c. with an overall yield of 12.5%. This is two to three times greater yields than previously obtained and current yields are getting as high as 25%. The synthetic gramicidin gave one single peak on HPLC with retention time exactly equal to Val^1-GA in the natural gramicidin mixture. The CD spectra of the synthetic and the natural Val^1-GA compare very well. The synthetic GA was characterized and compared with the natural gramicidin by ^{13}C n.m.r. spectroscopy and the Leu^{12,14} carbonyl carbon resonance assignment was made. The amino acid analysis, after hydrolyzing the sample with 4-N methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole, gave correct amino acid ratios with complete recovery of tryptophan but for the low valine value. The peptide sequence D-Val^6-Val^7-D-Val^8 was noticed to be resistant for complete hydrolysis under the conditions used. The single channel conductances of the synthetic GA were compared with those of the natural Val^1-GA in lipid bilayer membranes and shown to be indistinguishable, including a most probable conductance and a dispersion of lower conductance states with lesser probability. The mean single channel lifetime for channels whose conductance fell within the main peak group was 3.0 s.

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