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Total Synthesis and Ionophoric Behaviour of a Gramicidin A Analogue.

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Abstract : A new gramicidin A analogue (GAA) containing a C-terminal β-alaninamide group instead of the natural ethanolamine moiety has been synthesized on a polyacrylic resin and examined after its incorporation into a black lipid membrane (BLM). Preliminary conductance data show that this peptide and the natural gramicidin both display the same ionophoric activity.

Linear gramicidins are hydrophobic antibiotic natural peptides which, when incorporated in biological membrane, are able to form transmembrane channels permeable to alkaline metal ions[1]. According to the well-known Urry structure[2], head to head aggregation (between the formyl groups) of two gramicidin A molecules (Figure 1), stabilised by 22 intramolecular and 6 intermolecular H-bonds will result in the formation of a pore. The peptide chains are arranged in a so-called β-DL helix allowing all the lateral chains to remain inside the membrane lipid phase.

HCO-Val-Gly-Ala-(D)Leu-Ala-(D)Val-Val-(D)Val-[Trp-(D)Leu]$_3$-Trp-NH-C_2H_4-R
R=OH Natural gramicidin A R= $CONH_2$ Gramicidin analogue (GAA)

Figure 1

In order to show how the ethanolamine group located at the pore entry affects the conductance, as postulated by theoretical calculations[3], we synthesized a new analogue (GAA) in which the C-terminal OH group of ethanolamine was replaced by an amide function (Figure 1). Solid phase peptide synthesis methodology was applied in the construction of this peptide amide.

Owing to the presence of non polar residues in the GAA molecule, a polyacrylamide resin, such as Expansin® resin[4], showing good swelling capacity in DMF, has been selected as the best system to avoid chain peptide structure formation during the stepwise peptide elongation. In addition, a strategy based on the temporary Fmoc-protection of the amino group and allowing mild acidolysis for the final peptide cleavage from the support, leading directly to the required C-terminal amide group of the peptide, has been applied.

For this purpose N-Fmoc 4-carboxylatomethoxy 4'-methoxy benzhydrylamine (linker proposed by Breipohl *et al*[5,6]) was directly anchored to the amino group (0.68 meq/g) of the Expansin® resin. The overall yield of the linker synthesis has been improved from 29% to 54% as compared to Breipohl's publication[6]. After Friedel-

Crafts reaction, the oxime derivative[7] was subjected to zinc reduction leading to the benzhydrylamine acetate[7] which was then protected by a Fmoc group instead of Breipohl's reduction of the keto group by sodium borohydride followed by a fluorenylmethyl urethane substitution (separation of the alcohol from the remaining ketone was very difficult, giving a low overall yield).

After cleavage of the Fmoc-protecting group from the handle with a 20% piperidine solution, the coupling yield of Fmoc β-alanine with the linker was low (<50%) even when using HBTU. In order to decrease the steric hindrance, mainly responsible for this low yield, we inserted ε-amino caproic acid as a spacer between resin and handle. The coupling of Fmoc β-alanine then became quantitative.

This ε-amino caproic acid spacer can also be used as an internal reference to measure the overall yield of the peptide synthesis, by HPLC examination of the solution resulting from the acidic hydrolysis of the supported peptide.

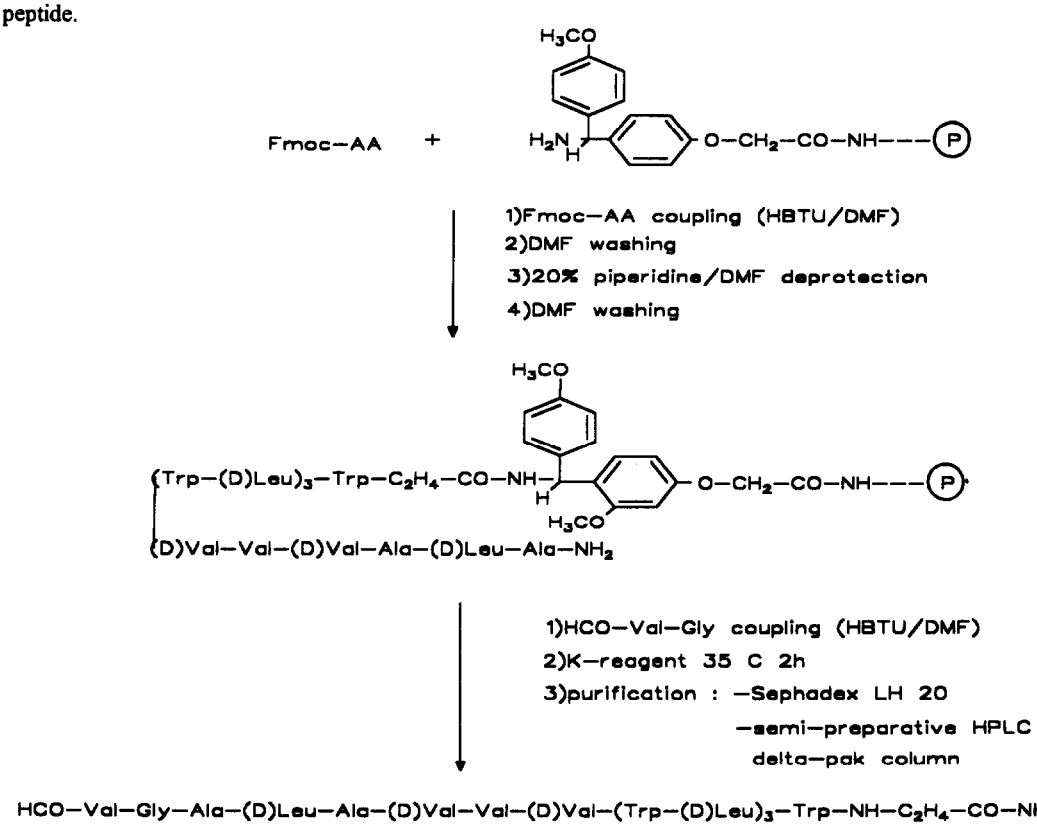


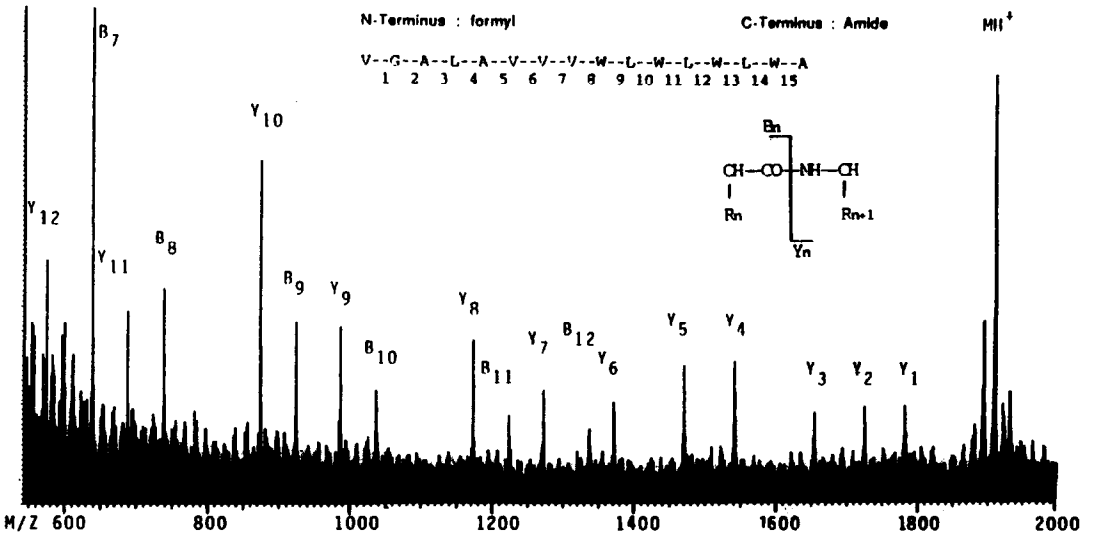
Figure 2

The solid phase peptide synthesis (Figure 2) was carried out using a half-automated synthesizer (Dupont Vega 1000). The fourteen first aminoacids were coupled according to a stepwise procedure using temporary Fmoc protection and HBTU as coupling reagent. The last fragment HCO-Val-Gly was previously prepared[8] and then directly anchored.

The crude peptide was then cleaved from its support by smooth acidolysis at 35°C for 2 hours using K-reagent[9] (thioanisol 5%; ethanethiol 2.5%; water 5%; TFA 85%). The cleavage was performed in good yield (85% based on the ε-amino caproic acid resin loading).

Further chromatographic purifications including gel filtration (LH-20 sephadex dextran/methanol) followed by semi preparative HPLC (Delta-Pak 5C18 Waters column, methanol/pH 7 phosphate buffer 78/22 V/V at 15ml/min, t_R=12 min) gave a purified peptide in 78% yield after lyophilisation. The HPLC-purified GAA (mp = 167-170°C) showed only one peak in analytical HPLC.

The aminoacid analysis as well the ^1H NMR spectrum and the FAB^+ mass spectrum (Figure 3) were in agreement with the expected formula of GAA.



C-terminal fragments (m/z) : Y_1 : 1782.0; Y_2 : 1725.9; Y_3 : 1653.8; Y_4 : 1541.8; Y_5 : 1469.7; Y_6 : 1370.7; Y_7 : 1271.6; Y_8 : 1172.6; Y_9 : 987.3; Y_{10} : 873.3; Y_{11} : 687.3; Y_{12} : 574.2

N-terminal fragments (m/z) : B_{12} : 1336.6; B_{11} : 1222.7; B_{10} : 1036.6; B_9 : 923.3; B_8 : 737.3; B_7 : 638.2

Figure 3 : FAB^+ mass spectrum of GAA[10]

Ionophoric activity of GAA : preliminary experiment[11].

This peptide, when incorporated in a lipid bilayer (glycerol monooleate in hexadecane) was able to generate transmembrane channels permeable to alkaline ions.

The resulting conductance arising from ion transportation between the two half cells was nearly the same in respect of pore life-time and current intensity as with natural gramicidin A. At 10^{-10} to 10^{-12} M concentration, either gramicidin A or the new GAA analogue gave rise to single channel events in 1M CsCl medium (Figure 4).

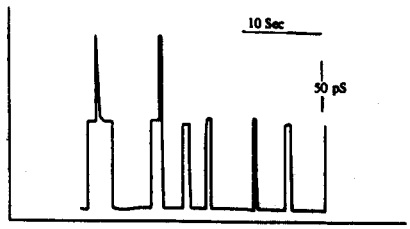


Figure 4 : Single-channel conductance of GAA

The discrete detectable conductance at the molecular level (82 pSiemens) corresponded to a single open channel for each event.

When several channels are simultaneously activated, there is a stepwise increase of the conductance during the life time of each individual channel.

In conclusion, gramicidin C-terminal modification by an amide group appears to play no role in the conductance properties of this peptide. The same conclusions have already been drawn for both desethanolamine-methylamine gramicidin[12] and also gramicidins which we have modified by carboxylic acid esterification[13].

Abbreviations: AA = amino acid residue, Fmoc = fluorenylmethyloxycarbonyl, TFA = trifluoroacetic acid, GAA = [Desethanolamine-β-alaninamide]gramicidineA, HBTU = 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.

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benzhydrylamine derivative acetate : mp=83-85°C, ^{1}NMR ($DMSOd_6$) δppm CH_3 at 1.75, two OCH_3 at 3.68 and 3.75, CH_2 at 4.71, CH at 5.82
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