Int. J. Peptide Protein Res. **43**, 1994, 10–18
Printed in Belgium – all rights reserved

INTERNATIONAL JOURNAL OF
PEPTIDE & PROTEIN RESEARCH
ISSN 0367-8377

Theoretical conformational analysis and synthesis of analogues of the heptapeptide antibiotic K-582 A

J.-L. DE COEN[1], B. WATHELET[2], F. DEMEUSE[2], C. MAYON[2], M. CULOT[3] and J. BRAKEL[3]

[1] *General Chemistry 1, Free University of Brussels, Brussels, Belgium,* [2] *Organic and Biological Chemistry and* [3] *Microbiology, Gembloux Faculty of Agronomic Sciences, Gembloux, Belgium*

Received 20 July 1992, accepted for publication 26 April 1993

A detailed theoretical conformational analysis of the linear heptapeptide antibiotic [Arg²]K-582 A (Arg-Arg-D-Orn-Thr-D-Orn-Lys-D-Tyr) was carried out. The results of the computer simulation suggest that the linear peptide has a high propensity to fold in solution into a quasi-cyclic conformation in equilibrium with π(L–D) helices. The synthesis of two inactive analogues with an L-Lys in place of D-Orn³ or D-Orn⁵ confirms the importance of the proposed folding pattern for the occurence of the antimicrobial activity of K-582 A. © Munksgaard 1994.

Key words: K-582; molecular graphics; peptide antibiotic; peptide synthesis

K-582 A and B are two linear heptapeptides showing antibiotic activities which were isolated by Kondo *et al.* (1) in 1980 from a culture of *Metarrhizium anisopliae*. The sequences of these peptides, which were determined by Kawauchi *et al.* (2), show a characteristic alternation of L and D amino acid residues: Arg-Arg(OH)-D-Orn-Thr-D-Orn-X-D-Tyr [X being Lys for K-582 A and Arg(OH) for K-582 B].

The synthesis and the study of the biological activities of several analogues [structures modified in positions 1 and 2 by Mihara *et al.* (3)] revealed interesting properties. The deletion of Arg¹ as well as the replacement of Arg(OH)² by Lys or Orn led to peptides devoided of any antibiotic effect, while the removal of the hydroxyl group on Arg(OH)² gave rise only to a small reduction in the potency of the peptide. These preliminary studies of the relationship between structure and activity of the K-582 peptides show the importance of the guanidine groups on positions 1 and 2 and set the question of whether the presence of these groups is required to insure the stability of some biologically active conformer or alternatively is essential for the binding of the peptide to the membrane or to some hypothetical receptor.

With the aim of proceeding further with the study of the relationship between structure and activity of the K-582 peptides, we undertook a detailed theoretical analysis of [Arg²]K-582 A and several analogues, the main idea being to use the results of the computer simulations as a guide for the synthesis of new derivatives.

In order to test the activity of [Arg²]K-582 A, this peptide was synthesized by the solid-phase method and purified by HPLC. Two other peptides with an L-Lys at position 3 or 5 in place of D-Orn were also prepared to test the importance of the L–D alternation.

EXPERIMENTAL PROCEDURES

Computation strategy for the theoretical conformational analysis of [Arg²]K-582 A

Many theoretical methods have been developed for the analysis of peptide conformation (4). Because of the limitation on computer power, all methods require the introduction of simplifying approximations. The strategy used in the present study was described previously (5) and applied succesfully in a number of cases (6–8).

The principle of the method lies in the calculation of a probability distribution of conformers for an n-peptide based on the calculation of the conformational energy in the classical approximation:

$$P(\{\phi_i, \psi_i, \{\chi_i\}\}) = \frac{\exp\left(-E(\{\phi_i, \psi_i, \{\chi_i\}\})/RT\right)}{Z}$$

with $i = 1$ to n residues

$$Z = \Sigma_{(\phi_i, \psi_i, \{\chi_i\})} \exp\left(-E(\{\phi_i, \psi_i, \psi_i, \{\chi_i,\}\})/RT\right)$$

The conformational energy $E(\{\phi_i, \psi_i, \{\chi_i\}\})$ is calculated using empirical potential energy functions describing torsional, van der Waals, electrostatic and

hydrogen-bond interactions. The parameters used in this work are the same as the one fully described in preceeding studies (5–8). A complete list of these parameters can be obtained on request.

The basic idea of our approach lies on the splitting of the conformational energy into two terms

$$E(\{\phi_i, \psi_i, \{\chi_i\}\}) = E_{\text{loc}}(\{\phi_i, \psi_i, \{\chi_i\}\}) + E_{\text{int}}(\{\phi_i, \psi_i, \{\chi_i\}\})$$

giving rise to a factorisation of the probability

$$P(\{\phi_i, \psi_i, \{\chi_i\}\}) = P_{\text{loc}}(\{\phi_i, \psi_i, \{\chi_i\}\}) \times P_{\text{int}}(\{\phi_i, \psi_i, \{\chi_i\}\})$$

The first term E_{loc} includes the contributions of the interactions at the local level of each residue and is indeed a sum of n terms $E^i_{\text{loc}}(\phi_i, \psi_i, \{\chi_i\})$ which each depends only on the values of the torsional angles ϕ_i, ψ_i and $\{\chi_i\}$ defining the conformation of residue i:

$$E_{loc}(\{\phi_i, \psi_i, \{\chi_i\}\}) = \sum_{i=1}^{n} E^i_{\text{loc}}(\phi_i, \psi_i, \{\chi_i\})$$

For obvious reasons the calculation of E_{loc} is quite straightforward and can be carried out without any problems regarding computer time, taking steps for the variables as small as 20 °, giving rise to an approximated probability distribution

$$P_{\text{loc}} = \prod_{i=1}^{n} \frac{\exp(-E^i_{\text{loc}}(\phi_i, \psi_i, \{\chi_i\})/RT}{Z^i_{\text{loc}}}$$

$$Z^i_{\text{loc}} = \Sigma_{\phi_i, \psi_i, \{\chi_i\}} \exp(-E^i_{\text{loc}}(\phi_i, \psi_i, \{\chi_i\})/RT$$

The second term E_{int} includes the contributions of the interactions between residues, and its calculation using steps as small as 20 ° for the variables would require extremely lengthy computer time as soon as the peptide length n reaches 5 or 6 residues. To overcome this difficulty, we proposed to calculate first the E_{int} terms only for a set of typical conformers choosen in the (ϕ, ψ) regions where P_{loc} is not negligible ($P_{\text{loc}} > 10^{-4}$). This first scan provides a set of conformations of low energy which are afterwards refined to give conformers of minimal energy.

The refinement procedure used in this work to minimize the conformational energy is the one included in the computer graphics software BRUGEL (9). The values of the geometrical and energetical parameters used in BRUGEL are very similar to the one included in CHARM (10). These values are available on request.

Peptide synthesis

Materials. Commercial protected amino acids and chloromethyl resin 1.08 meq./g were obtained from Bachem (Bubendorf, Switzerland).

Acetylation of α-NH_2. 20 mL of a solution of 20% acetic anhydride and 2% DIEA in CH_2Cl_2 were added to the peptide-resin. After 10 min the peptide-resin was washed three times with CH_2Cl_2.

Cleavage of the peptide from Merrifield resin. 1 g of peptidyl-resin was cleaved by 10 mL anhydrous HF (highly toxic) during 1 h in the presence of 1 g *p*-cresol as scavenger. After evaporation of HF, the peptide was precipitated with diethyl ether, filtered and extracted with 5% AcOH. The solution was then lyophilized.

Analytical and preparative reversed-phase HPLC. The instrument was a Spectra Physics 8800. The analytical column (12.5 × 0.4 cm) was packed with Nucleosil 120-3C_{18}, 3 μm (Macherey Nagel 720040), and the preparative column (50 × 1 cm) was packed with Nucleosil 100-5C_{18}, 5 μm (Macherey Nagel 71213). The analytical loop contained 20 μL and the preparative 1000 μL.

Analytical elution was accomplished by a binary buffer system. Solvent A was 0.07% TFA in water and solvent B was 0.05% TFA in CH_3CN. The peptides were eluted with a linear gradient (from 0 to 50% of solvent B in 30 min). The flow rate was 0.7 mL/min at room temperature. Peptides were detected at 215 nm with a Spectra Physics 100 variable ultraviolet detector. Preparative elution was accomplished with the same

TABLE 1

Determination of the minimal inhibitory concentration (MIC) in μg/mL

Tested organisms	MIC (μg/mL)		
	[Arg^2] K-582 A	[Arg^2, Lys^3] K-582 A	[Arg^2, Lys^5] K-582 A
Candida tropicalis MUCL29817	500	1000	1000
Rhodotorula mucilaginosa MUCL27809	6	50	50
Bacillus subtilis MicrobioGembloux	200	200	200
Staphylococcus aureus ATCC29213	1000	1000	1000
Micrococcus luteus ATCC4698	1000	1000	1000
Mycobacterium smegmatis CNCM7326	1000	200	200
Pichia jardinii MUCL27719	6	1000	1000

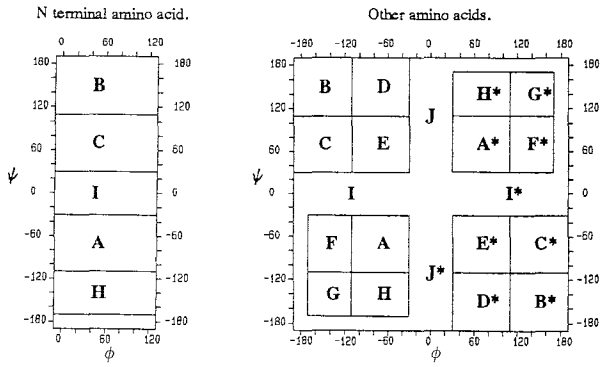

FIGURE 1
Ramachandran (ϕ, ψ) plot with stereochemical code.

type of gradient adapted to the retention time of the peptide to purify and a flow rate of 2.5 mL/min.

Biological tests

Sub-culture of colonies. Colonies were taken with a platinum thread and sub-cultured on plate count agar (PCA) for bacteria or on solid potato dextrose agar (PDA) for yeasts and fungi, and incubated for 3 days at 30 °C with the exception of *Mycobacterium smegmatis* and *Staphylococcus aureus*, which were incubated at 37 °C.

Determination of minimal inhibitory concentration. Different concentrations of peptide were tested: 1000, 500, 250, 200, 100, 50, 25, 12, 6, 1, 0.5 and 0.25 μg/mL. In the well of a microtitration plate 8 × 12 the following solutions were successively deposited: 50 μL of the sterile peptide solution, 140 μL of nutrient broth (equivalent to PCA) or 140 μL of malt extract broth (equivalent to PDA) and 10 μL inoculum (10^3 cells/mL). The plates were covered with a plastic sheet and placed in an oven at 30 °C. After inoculation, culture development was compared visually with the culture of the same strain without the peptides.

RESULTS AND DISCUSSION

Synthesis of the linear peptides [L-Arg²]K-582 A and analogues with L-Lys in positions 3 and 5

The peptides were synthesized on a Merrifield resin with a semi-automatic synthesizer. The first amino acid

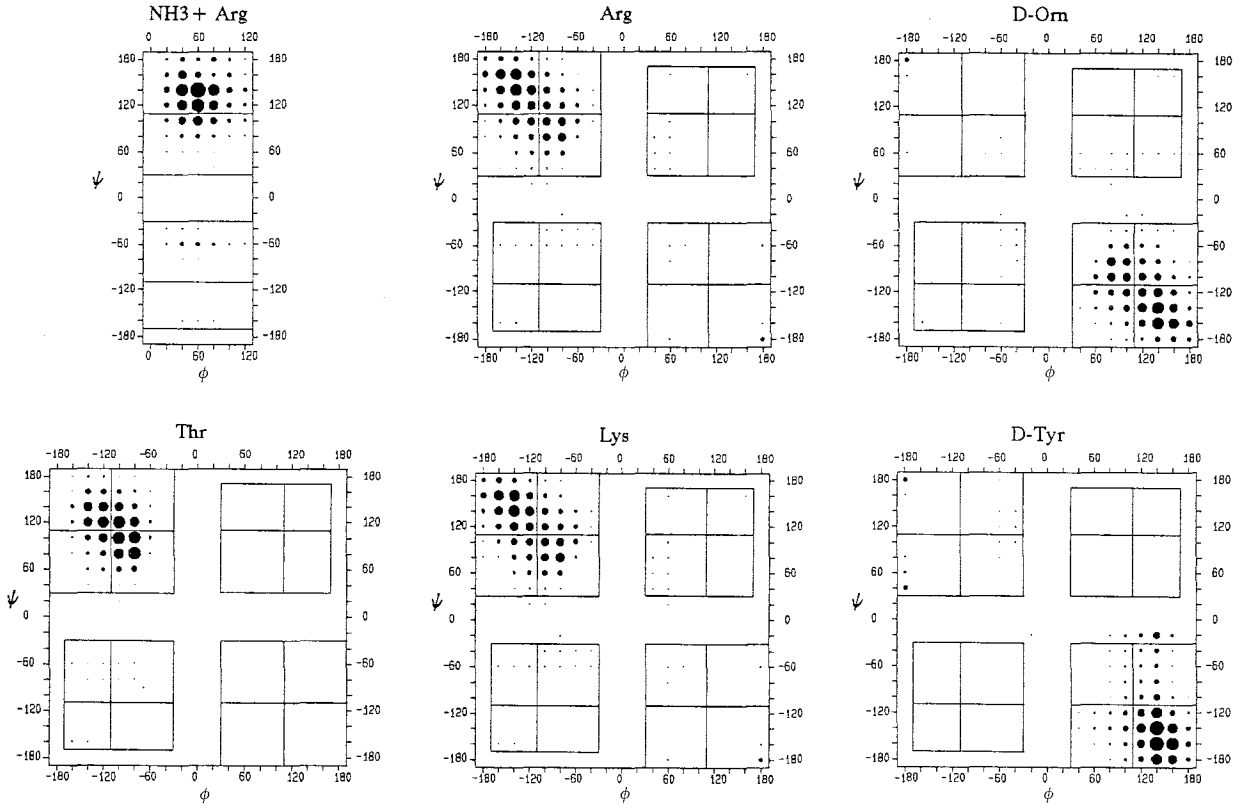

FIGURE 2
Ramachandran (ϕ, ψ) plot of the probability distribution $P_{i,\mathrm{loc}}(\phi_i, \psi_i)$ for each residues of [Arg²]K-582 A.

TABLE 2

Probability distribution $P_{loc}(R)$ in % of the different regions for the six different residues of [Arg²]K-582 A

Regions	NH_3^+ Arg	Arg	D-Orn	Thr	Lys	Tyr-COO^-
A	2.3754	0.1071	0.0667	0.0307	0.1318	
B	81.4332	54.0649	0.8409	28.1595	52.6099	1.0596
C	16.1838	11.7374	0.0159	9.6639	11.5336	1.2260
D		12.7807	0.0055	22.9154	12.5602	0.2072
E		20.3902	0.0298	39.1935	22.1047	0.0448
F		0.0274	0.0001	0.0290	0.0296	
G		0.0038	0.0016	0.0030	0.0042	
H	0.0077	0.0002	0.0015	0.0001	0.0002	
I	0.0000	0.0137	0.0011	0.0031	0.0174	0.0013
J		0.0005	0.0001	0.0000	0.0005	
A*		0.0616	0.1524	0.0000	0.0755	
B*		0.7709	53.1031	0.0009	0.8792	77.4477
C*		0.0123	12.4713	0.0000	0.0137	8.4933
D*		0.0064	11.5157	0.0000	0.0064	6.5715
E*		0.0190	21.7401	0.0000	0.0294	0.9246
F*		0.0000	0.0331	0.0000	0.0000	
G*		0.0016	0.0038	0.0000	0.0015	
H*		0.0019	0.0002	0.0000	0.0017	
I*		0.0008	0.0167	0.0000	0.0012	4.0238
J*		0.0000	0.0005	0.0000	0.0000	0.0002

TABLE 3

Values of ϕ and ψ angles (in degrees) chosen for each residue of [Arg²]K-582 A, as representative of the various populated regions (see Fig. 2)

Residue	Stereochemical code	ϕ	ψ
NH_3^+ Arg	b	60	140
	a	− 60	− 40
Arg	b	− 140	140
Thr	c	− 140	80
Lys	d	− 80	140
	e	− 80	80
	a*	60	40
	a*	60	40
	b*	140	− 140
D-Orn	c*	140	− 80
	d*	80	− 140
	e*	80	− 80
	a	− 60	− 40
D-Tyr COO^-	b*	140	− 140

TABLE 4

Ten lowest-energy conformations obtained after minimization

No.	Label	Conformation	Energy	Distance N_1–C_7 (Å)
1	+	c e e* c d* i* b*	− 44.3	3.20
2	*	c e e* e d* i* b*	− 42.9	3.24
3	◇	b b c* e e* e b*	− 41.5	6.71
4	⊕	b e e* e e* b b*	− 40.0	3.12
5	▽	b c i* e e* b b*	− 39.1	8.14
6	●	b b d* e e* i d*	− 39.1	10.87
7	∅	b c d* e i* e c*	− 39.0	3.62
8	⨯	b f e* e b* e b*	− 38.6	5.70
9	#	b d i e e* b b*	− 38.6	4.99
10	⊗	b b e* e e* c c*	− 37.7	9.25

(Boc-D-Tyr(Z)-OH) was coupled to the resin as a cesium salt (11).

Before each coupling, the α-NH_2 group of the growing peptide was deprotected with a solution of 50% TFA in CH_2Cl_2 and neutralized with a solution of 5% DIEA in CH_2Cl_2. The subsequent amino acid, with a Boc group on α-NH_2 and a lateral protection, was activated with DCC and HOBt, pre-incubated for 10 min (20 min for Boc-L-Arg(Z)-OH) and allowed to react with the peptide. The excess of reagent was 2.5 fold. The reaction was controlled by a Kaiser test (12).

After the addition of the last amino acid, the peptides were cleaved from the resin and deprotected by HF acid, lyophilized and purified by reversed-phase HPLC. The synthesis yield was 92% for [Arg²]K582-A, 85% for [Arg²][Lys³]K582-A and 87% for [Arg²][Lys⁵]K582-A. No special problems were observed. These peptides had a retention time of, respectively, 12.15, 12.17 and 12.15 min in analytical HPLC. After gel filtration on TSK HW-40(S) and preparative HPLC, the peptides had a purity greater than 98%.

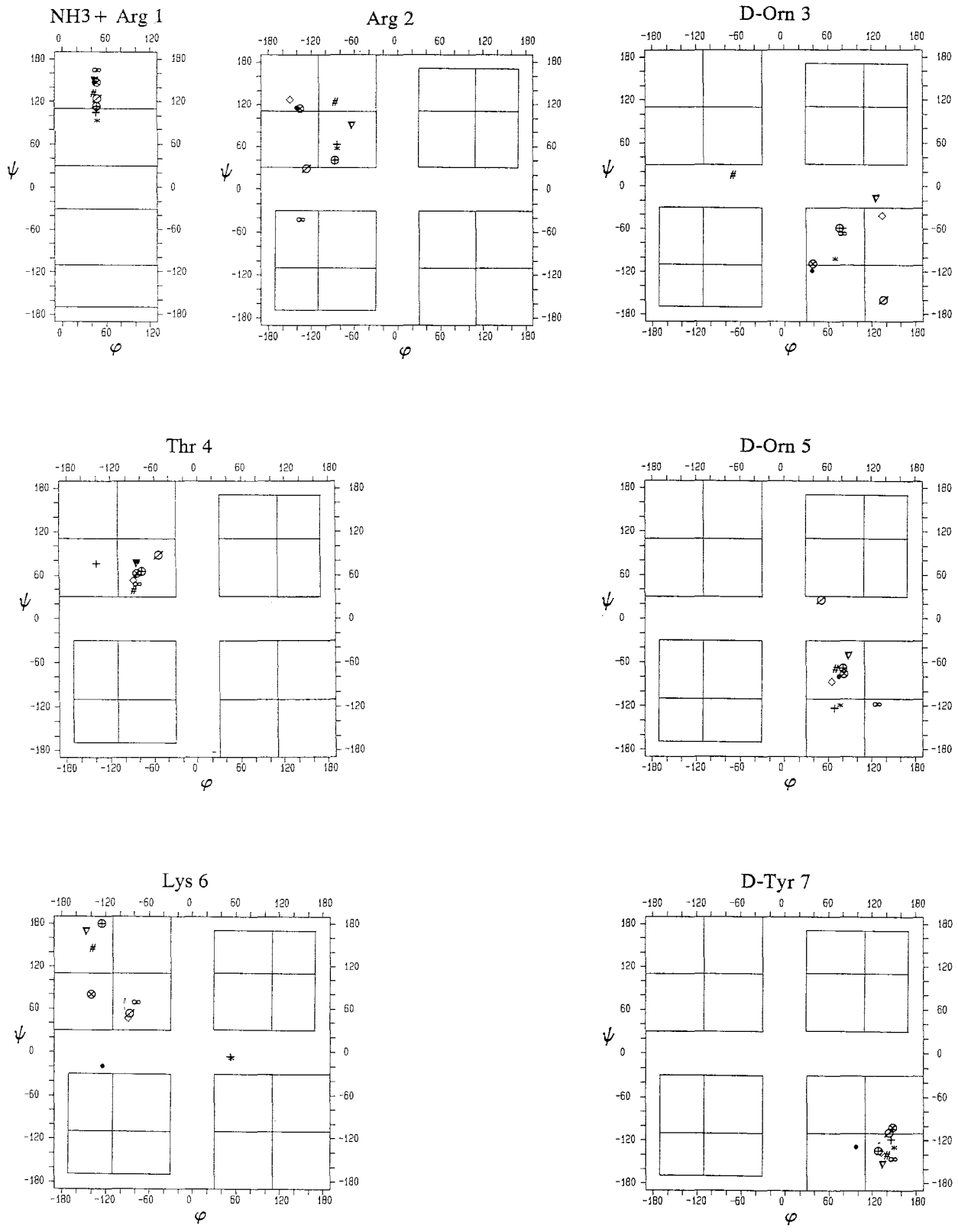

FIGURE 3
Plot of ϕ and ψ angles for ten conformers of minimal energy.

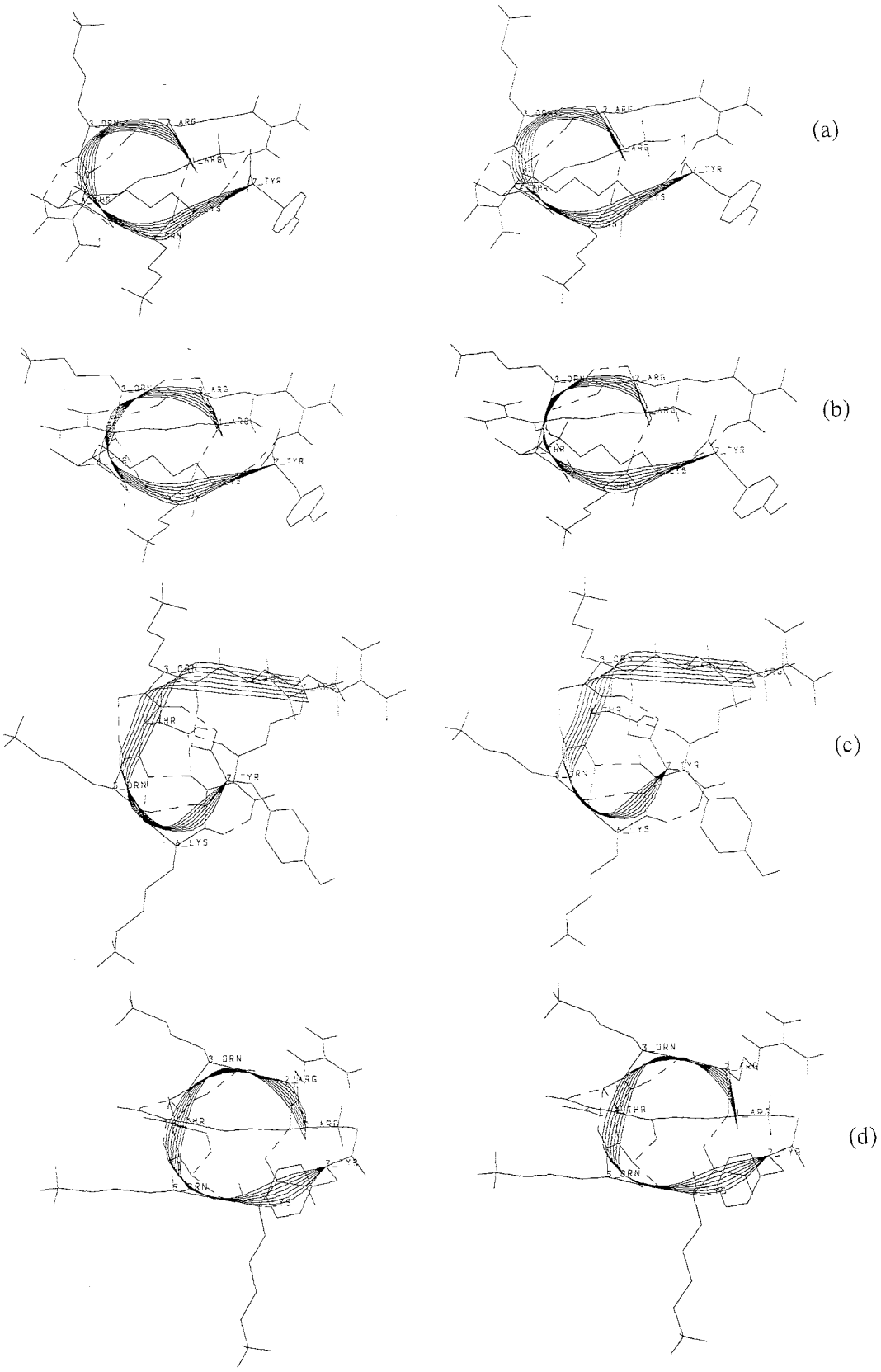

FIGURE 4a,b,c,d

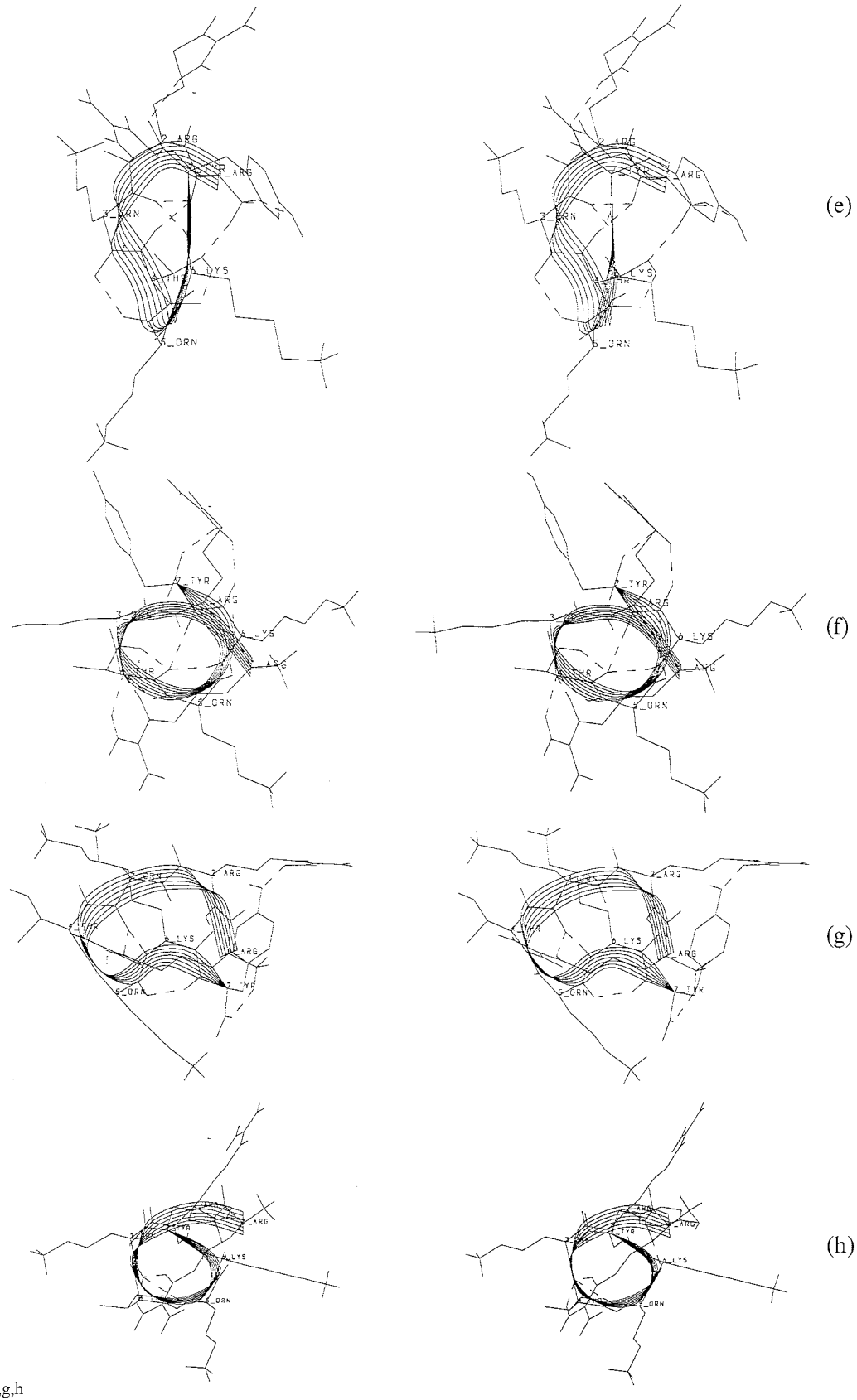

FIGURE 4e,f,g,h
Stereo views of eight conformers of minimal energy for [Arg^2]K-582 A (nos. 1–7 and 10 in Table 4).

Biological activity of the three synthetic peptides

The antimicrobial spectra of the three peptides were determined using seven strains of micro-organisms (Table 1). The results shown indicate that these peptides are mostly effective against yeast and fungi, and rather inactive against bacteria.

These data are in general agreement with the results of previous studies (1, 3). [Arg^2]K-582 A appears to be the most active peptide. The main difference in activity between [Arg^2]K-582 A and K-582 A concerns *Candida tropicalis*. The analogue shows a very low activity compared to that of the native peptide.

Computer simulation

The results of the calculation of the probability distribution P^i_{loc} for each residue of the peptide are given on Ramachandran (ϕ,ψ) plots (Figs. 1 and 2) and summarized in Table 2. The values of the angles (ϕ, ψ) associated with a probability P_{loc} (ϕ, ψ) $\geq 10^{-4}$ are identified by a circle with a surface proportional to the probability. These results indicate that there exist $55 \times 82 \times 80 \times 53 \times 80 \times 83 \times 60 \approx \pm 10^{13}$ backbone conformers with $P^i_{loc} > 10^{-4}$ for each residue. The calculation of P_{int} for all these conformations clearly being impossible, we chose to test the importance of the E_{int} terms by calculating a relative probability distribution for the $1 \times 6 \times 6 \times 6 \times 6 \times 6 \times 1 = 7776$ typical backbone conformers defined by the (ϕ, ψ) angles given in Table 3. The results of this calculation indicate that only 91 conformers represent more than 99% of the total probability. This pool of 91 conformers was taken as representative of highly probable conformations and used to generate models of minimal energy. The ten lowest energy conformations obtained after refinement are listed in Table 4. The (ϕ, ψ) angles defining the backbone folding of these models are plotted on Ramachandran (ϕ, ψ) maps for each residue in Fig. 3. The folding adopted by the main chain as well as the side chains for several models is illustrated by the stereo views in Fig. 4.

The results of the calculation of local interactions reported in Fig. 2 and Table 2 indicated that the preferred conformations of [Arg^2]K-582 A are labelled BBB*EB*BB*, BBB*BB*BB*,... These conformations are quasi-cyclic structures, in contrast to the preferred conformers of peptides containing only L-residues where local effects favour extended conformers such as BBBBBBB, which is the β-pleated strand. The difference in the general folding pattern predicted for short all L and LD peptides is illustrated in Fig. 5.

The main folding of [Arg^2]K-582 A into quasi-cyclic structures being favoured by local effects, one may expect that the net results of long-range interactions would be to bring further stabilization to a few typical conformers. The results shown in Table 4 and Fig. 4 seem to indicate that this is indeed the case.

Models 1, 2, 4 and 7 (see Table 4 and Figs. 4a, b, d and g) are quasi-cyclic structures, the distances between the N atom of the amino terminal and the C atom of the carboxyl terminal being, respectively, 3.20, 3.24, 3.12 and 3.62 Å (Table 4). In model 3, which adopts a sigma-like shape, the carboxy terminal is hydrogen-bonded to NH of Orn^3 and Thr^4, giving rise to a quasi-cycle including residues 3–7. Model 5 is stabilized by several hydrogen bonds, including donors and acceptors from the main as well as the side-chains. The folding of the backbone in this case seems to be not far from that of the right-handed π(L–D)-helix (13). Models 6 and 10 are very similar and can be classified as models of the family of the left-handed π(L–D)-helix.

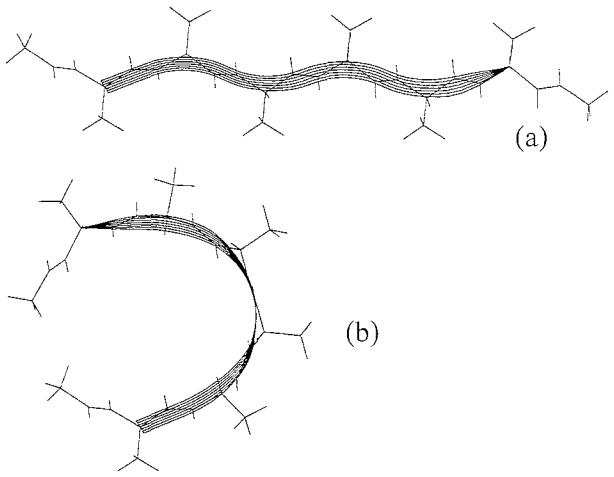

FIGURE 5
Typical low-energy conformers of short all-L (a) or L–D (b) peptides.

Among the quasi-cyclic structures 1, 2, 4 and 7, models 1 and 2 are very similar as far as the folding of the main chain is concerned (Figs. 4a and 4b) and labels + and * on the (ϕ,ψ) plots in Fig. 3). These two models indeed differ only in the side-chain arrangements at the level of Orn^3. In both cases the side-chain of Arg^1 is hydrogen-bonded to the CO of Orn^3 and by the way the aliphatic moiety of this side chain run over the ring drawn by the backbone. The same feature being observed in other models, the results of our analysis strongly support the idea of an important structural role for Arg^1.

Another common caracteristic of most of the models (Fig. 4) is the presence of the Arg^2 and D-Tyr^7 side chains in the same region of the space and this also could be related to important binding properties of the peptide. The other basic side-chains D-Orn^3, D-Orn^5 and Lys^6 are for most of the models not involved in intramolecular interactions and thus free for being solvated in water. This feature is particularly evident in models 6 and 10 which belong to the left π(L–D)-helical family.

In summary one can say that the results of the theoretical conformational analysis carried out in this work suggest that the heptapeptide [Arg^2]K-582 A exists in

'solution' as an equilibrium between a few quasi-cyclic conformations and folded conformers reminiscent of right and left handed π(L–D) helices. The presence of the left-handed π(L–D) helix in aqueous solution was already suggested by Mihara *et al.* (3) from the observation that the CD spectrum of K-582 A is analogous to the one observed for L–D peptides such as Gramicidin A which are known to adopt the π(L–D)-helix folding (13).

In the computer simulation carried out in this work, the solvent is not explicitly taken into account but part of its effects is introduced through the use of a dielectric constant, which is taken here equal to the distance between atoms in Å, and through the screening of fully charged groups which are considered to bear a net charge divided by 3.

The values of the energy terms associated with the different conformers listed in Table 4 could of course be quite dependent on the choice of these and other energetical parameters. These energetical values must thus be considered with great care. More confident results of the theoretical analysis lie in the suggested topographical properties of the various families of conformers. There are topographical properties which are specific to the sequence of [Arg^2]K-582 A that could explain the possible specific binding of this molecule to membranes or some other macromolecular receptors. Another interesting potentiality offered by the theoretical analysis concerns the possibility of establishing a correlation between the topographical properties of analogues and their biological activities. For example, it is most likely that the replacement of D-Orn^3 or D-Orn^5 residue by L-Lys gives rise to inactive analogues (Table 1) because the π(L–D) helices as well as the quasi-cyclic conformations are destabilized by such substitutions.

We are now attempting to confirm this hypothesis by synthesizing analogues which could favour the presence of the π(L–D) helices or some of the quasi-cyclic conformations (14).

ACKNOWLEDGMENTS

J.-L. De Coen is a research associate at the Fonds National de la Recherche Scientifique.

REFERENCES

1. Kondo, S., Meguriya, N., Moji, H., Aota, T., Miura, K., Fujii, T., Hayashi, I., Makino, K., Yamamoto, M. & Nakajima, N. (1980) *J. Antibiotics* **33**, 533–541
2. Kawauchi, H., Tohno, M., Tsuchiya, Y., Hayashida, M., Adachi, Y., Mukai, T., Hayashi, I., Kimura, I. & Kondo, S. (1983) *Int. J. Peptide Protein Res.* **21**, 546–554
3. Mihara, H., Aoyagi, H., Yonbezawa, H., Kuromiza, K. & Izumiya, N. (1985) *Int. J. Peptide Protein Res.* **25**, 640–647
4. Zimmerman, S. (1985) in *The Peptides: Analysis, Synthesis and Biology* (Hruby, V.J., ed.), vol. 7, pp. 165–212, Academic Press, New York
5. Ralston, E. & De Coen, J.-L. (1974) *J. Mol. Biol.* **83**, 393–410
6. De Coen, J.-L. & Ralston, E. (1977) *Biopolymers* **16**, 1929–1943
7. Humblet, C. & De Coen, J.-L. (1981) *Int. J. Biol. Macromol.* **3**, 37–45
8. Tourwe, D., De Coen, J.-L., Hallenga, K. & Van Binst, G. (1984) *Int. J. Peptide Protein Res.* **23**, 84–93
9. Delhaise, P., Bardiaux, M. & Wodak, S. (1984) *J. Mol. Graphics* **2**, 103–106
10. Brooks, B., Bruccoleri, R., Olafson, B., States, D., Swaminathan, S. & Karplus, M. (1983) *J. Comput. Chem.* **4**, 187–217
11. Gisin, B. (1973) *Int. J. Peptide Protein Res.* **56**, 1476–1482
12. Kaiser, E., Colescott, R., Bossinger, C. & Cook, P. (1970) *Anal. Biochem.* **117**, 147–157
13. Urry, D., Goodall, M., Glickson, J. & Mayers, D. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 1907–1911
14. De Coen, J.-L., Demeuse, F., Mayon, C. & Wathelet, B. (1991) Communication at the FNRS-NFWO contact group meeting, November 15, Brussels (unpublished results)

Address:

J.-L. De Coen
Chimie Générale 1
Université Libre de Bruxelles (CP160/16)
Av. F.D. Roosevelt 50
B-1050 Bruxelles
Belgique
Tél.: 02/650.35.87
Fax: 02/648.89.54