Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Crystal Structure of a Spin-Labelled, Channel-Forming, Alamethicin Analog

M. Crisma, C. Peggion, C. Baldini, E.J. MacLean, N. Vedovato, G. Rispoli, C. Toniolo

1. X-RAY DIFFRACTION

1a. Data collection, structure solution and refinement

Crystals suitable for X-ray diffraction were grown from a methanol solution by slow evaporation and are stable in the absence of mother liquor. A crystal in the form of a thin plate, 0.40 × 0.06 × 0.02 mm^3 in size, was mounted on the end of a two-stage glass fibre with perfluoropolyether oil. Diffraction data were collected at 150 K using synchrotron radiation (λ = 0.6868 Å) with a Bruker Nonius (Madison, WI) APEX II CCD area-detector diffractometer at the microcrystal diffraction facility on station 9.8 of the Synchrotron Radiation Source, CCLRC Daresbury Laboratory. Data collection nominally covered a sphere of reciprocal space by a series of ω-rotation exposure frames with different crystal orientation ϕ angles. Reflection intensities were integrated using standard procedures,[1] allowing for the plane-polarised nature of the primary synchrotron beam. Corrections were applied semiempirically for absorption and incident beam decay. Unit cell parameters were refined from the observed ω angles of all strong reflections in the complete data set. At first, data processing suggested a monoclinic cell in space group C2, with parameters *a* = 39.767(4), *b* = 60.576(7), *c* = 32.655(3) Å, and β = 116.091(2)°. Cell volume and density considerations (combined with the stability of the crystals in the absence of mother liquor which, in turn, suggested a very low, if any, solvent content), indicated the occurrence of six independent peptide molecules in the asymmetric unit (about 900 light, non-H atoms), a non-trivial task for structure solution. The structure was eventually solved *ab initio* by seeding the dual-space direct methods of the SHELXD program[2] by a 6D search (through random translation and rotation) for a rigid fragment consisting of the backbone and C^{β} atoms of residues 1-14 of one of the three independent molecules present in the X-ray structure of natural alamethicin F30.[3] The solution allowed the location of 781 atoms in more or less large molecular fragments. Through cycles of conjugate gradient least-squares refinement, by application of the SHELXL-97 program,[4] the six independent molecules could be built by recovering the position of the remaining atoms from difference Fourier maps. The size of this structure would be the second largest, after the 1033-atom structure of feglymycin reported by Sheldrick,[5] among equal-atom structures solved *ab initio*. However, the refinement turned out to be unsatisfactory, giving at best R_1 = 0.238 [on F ≥ 4σ(F)], with many atomic displacement parameters becoming non-positive definite when anisotropically refined. In this model the six peptide molecules can be described as three pairs, each composed of two conformationally distinct molecules. The second and third pair of molecules are related to the first pair by pseudosymmetry operations, namely *x*, -1/3+*y*, *z*, and ½-*x*, -1/6+*y*, -*z*, respectively. This observation prompted a closer inspection of the data, which revealed that the reflections with $k \neq 3n$ are consistently weaker than the others, and the corresponding layers of diffraction spots are much less ordered. Taken together, the findings reported above may suggest

the possibility of non-merohedral twinning of the crystals, with one prevailing twin domain. However, as attempts to index the weak data independently were unsuccessful, this issue could not be further clarified. On these bases, data processing was then performed on a monoclinic cell, again in space group C2, but with the *b* axis reduced to one third of that previously assumed, i.e. with unit cell parameters *a* = 39.767(4) Å, *b* = 20.192(3) Å, *c* = 32.655(3) Å, β = 116.091(2)°, and V = 23549(5) Å^3. The corresponding dataset is composed of 68928 reflections collected up to 2θ = 42.4° (0.95 Å resolution), of which 28257 are unique, with R_{int} = 0.041.

In this smaller cell the structure was again solved *ab initio* with the SHELXD program as described above. The asymmetric unit is composed of two independent peptide molecules and three co-crystallized water molecules (empirical formula $C_{202}H_{334}N_{42}O_{59}$), for a total of 303 non-H atoms. The structure solution allowed the location of 285 atoms in three large peptide fragments. The model was completed through standard small-molecule techniques by recovering the positions of the remaining atoms from subsequent difference Fourier maps interleaved by cycles of refinement. Refinement was carried out by full-matrix block least-squares procedures on F^2, with all non-H atoms refined anisotropically, by application of the SHELXL-97 program. All data were used, except for a 5% fraction which was reserved for R_{free} calculations. The hydroxyl group of the C-terminal Phol^{20} of molecule **A** is disordered. It was refined on two positions (atoms OT20 and OT'20) with population parameters of 0.70 and 0.30, respectively. Some disorder is also likely to occur at the level of the terminal atoms of some Glu(OMe) side chain, as suggested by their large displacement parameters [largest U_{eq} = 0.36(2) Å^2]. However, despite extensive effort, the data did not support a viable model to unravel such disorder satisfactorily. A planarity restraint was imposed on the phenyl ring of the Phol residues. Restraints were also applied to most of the bond distances and bond angles, as well as to the anisotropic displacement parameters of some side-chain atoms. H-atoms of the two peptide molecules were calculated at idealized positions and refined using a riding model. The positions of five (out of six) H-atoms of the three co-crystallized water molecules were recovered from a difference Fourier map but they were not refined. Refinement converged to R_1 = 0.117 [on F ≥ 4σ(F)], wR_2 = 0.305 (on F^2, all data). Goodness of fit on F^2: 1.081. Data / restraints / parameters: 26845 / 851 / 2725. Average U_{eq} = 0.108(4) Å^2. The values of the R_{free} factor before and after introduction of anisotropy, and at the end of refinement were 0.249, 0.183 and 0.134, respectively.

1b. Geometrical Details

The TOAC piperidine rings adopt a twist-boat conformation, namely $^2T_6$ in molecule **A,** while $^6T_2$ in molecule **B**. These two enantiomeric conformations are those largely prevailing for TOAC peptides and for other six-membered nitroxide derivatives as well, as a result of the combination of ring flattening

at the sp^2 nitrogen atom and tetramethylsubstitution at the vicinal carbon atoms. [6] The $^2T_6$ disposition of molecule **A** is that most commonly found for right-handed helical TOAC peptides,[6] whereas the occurence in molecule **B** of the less populated $^6T_2$ disposition might be related to the H-bonding of the nitroxide OD36 oxygen atom with a co-crystallized water molecule. The puckering parameters [7] [relative to the ring atom sequence N^{δ}-(pro-D)C^{γ}-(pro-D)C^{β}-C^{α}-(pro-L)C^{β}-(pro-L)C^{γ}] are Q_T = 0.654(9) Å, φ_2 = 268.1(8)° and θ_2 = 86.5(9)° for TOAC^{16}, and Q_T = 0.674(11) Å, φ_2 = 86.6(8)° and θ_2 = 88.3(8)° for TOAC^{36}.

The N^{δ}-O^{δ} bond length of the TOAC nitroxide group is 1.282(10) Å and 1.297(11) Å in molecules **A** and **B**, respectively, within the range observed for other nitroxide-containing compounds,[6] while significantly shorter than the typical length (1.39 ÷ 1.40 Å) for an (sp^2) N-O single bond.[8] This finding provides evidence that the integrity of the nitroxide free radical has been preserved throughout the various steps of the synthetic procedure. In molecule **A** the midpoint of the N^{δ}-O^{δ} nitroxide bond is located at 22.8 Å from N1, and at 9.8 Å and 8.3 Å, respectively, from O18 and O19. The corresponding distances in molecule **B** are 24.4 Å from N21, 9.5 Å from O38, and 8.0 Å from O39.

Molecule **A** is more sharply bent near the Pro^{14} residue than molecule **B**. The bending angles (calculated as in ref. [9]) are 32° and 25° for molecules **A** and **B**, respectively, very close to those of molecules I and II (33° and 31°), and molecule III (22°) of alamethicin F30.[3] The C^{α}(1) - C^{α}(20) distance is 28.6 Å in molecule **A**, while the corresponding C^{α}(21) - C^{α}(40) distance in molecule **B** is 29.4 Å.

2. ELECTROPHYSIOLOGICAL MEASUREMENTS

In order to test directly the pore-forming capability of alamethicin analogs under physiological conditions, a custom-made, computer controlled microperfusion system was employed to rapidly apply (and remove) the peptides under test onto a cell, recorded in whole-cell configuration, in which all the endogenous conductances were fully blocked. The isolated retinal rod outer segments (OS) of frog photoreceptor have been found particularly suitable to carry on this study, because of their large size and because the predominant endogenous membrane conductance are the light sensitive channels.[10,11] If the OS is illuminated, the light sensitive channels close, and under these conditions the OS membrane resistance recorded in whole-cell typically exceeded 0.5 GΩ, allowing the detection of the current produced by the peptides under study with a resolution of 1 pA in a bandwidth of at least 1 kHz.

Rod outer segments (OS) were mechanically isolated from the retina of the *Rana esculenta* (25-40 gr body weight) as described in detail elsewhere.[12] All manipulations were made in the dark using infrared illumination and an infrared viewer (Find-R-Scope, FJW Optical Systems, Palatine, IL, USA). The retina was "peeled" from an eyecup piece and was gently triturated in ~5 ml of Ringer, using a fire-polished Pasteur pipette to obtain the OS; the fluid drop containing the OS was then transferred to the recording

chamber. The OS on the microscope stage (TE 300, Nikon, Tokyo, Japan) were illuminated with an ultrabright infrared LED (900 nm) and viewed on a TV monitor connected to a contrast enhancement camera (Till Photonics, Planegg, Germany) coupled to the microscope.

OS were recorded using the whole-cell configuration of the patch-clamp technique under visual control at room temperature (20-22 °C), employing Axopatch 200B (Axon Instruments, Union City, CA, USA). The Ringer had the following composition: 115 mM NaCl, 3 mM KCl, 10 HEPES, 0.6 mM $MgCl_2$, 0.6 mM $MgSO_4$, 1.5 mM $CaCl_2$, 10 mM glucose (Osmolality 260 mOsm/Kg, buffered to pH=7.6; all chemicals were purchased from Sigma Chemical Co.). The current amplitude elicited by a -10 mV pulse in cell attached and during whole-cell recording was used to measure seal resistance and membrane resistance, respectively.

Once the whole cell recording was gained, the cell was aligned in front of a multibarrelled perfusion pipette that can be moved on an horizontal plane. Peptides were applied and removed in less than 100 ms by switching forth and back the OS from a stream of control perfusion solution (composition, in mM: 100 K^+, 1 Ca^{2+} and 10 HEPES; Osmolality 260 mOsm/Kg, buffered to pH=7.6 with KOH) to a stream containing the peptide (dissolved in the same perfusion solution). This strategy allowed to assess the dynamics of the pore formation and the possible reversibility of this process. Patch pipettes were filled with the same perfusion solution, in order to drive the current just with the holding voltage, that was typically –20 mV.

Peptides were dissolved in methanol to get a 100 μM stock solution; an aliquot of this stock was dissolved in the perfusion solution to get a final concentration of 1 μM, and used within 30 min. Control experiments proved that contamination by methanol of the perfusion solution (about 10 nl/ml) did not cause any non-specific membrane permeabilization. Recordings were filtered at 2 kHz via an eight-pole Butterworth filter (VBF/8 Kemo, Beckenham, UK), sampled on-line at 5 kHz by a Digidata 1322A connected to the SCSI port of a Pentium computer running the pClamp 9.0 software package (Molecular Devices, Sunnyvale, CA, USA), and stored on disk. Data were further low-pass filtered off-line at 200 Hz using a gaussian filter and analyzed using Clampfit (Molecular Devices). Figures and statistics were performed using SigmaPlot (Jandel Scientific, Sam Rafael, CA). The values in text and figures are given as means±SEM.

The permeabilization properties of the peptides were tested by means of the following protocol. The isolated rod outer segment (OS) was hold to a fixed voltage (-20 mV) and the membrane resistance was measured from the current deflection elicited by a -10 mV pulse superimposed to the holding voltage before peptide application. The peptide was then quickly applied (<100 ms) using the fast perfusion system described in the Methods. The OS was finally returned to the control solution (without the peptide) to assess a possible recovery of the current to, or toward, the 0 level, and the membrane resistance was measured again. The [$TOAC^{16}$, $Glu(OMe)^{7,18,19}$] alamethicin F50/5 analog at 1 μM

concentration permeabilized the OS, producing a current of -530±40 pA (n=6) at -20 mV, whereas the current produced by the [Glu(OMe)^{7,18,19}] analog was -750±90 pA; (n=6). The permeabilization induced by both peptides was fully reversible: the current returned to 0 level, and membrane resistance recovered its high value before peptide application within 20 s after peptide removal from external perfusion solution.

Supporting References

[1] SAINT version 7.06a, Bruker AXS Inc., Madison, Wisconsin (USA), **2004**.

[2] G. M. Sheldrick, H. A. Hauptmann, C. M. Weeks, R. Miller, I. Usón in *International Tables for Crystallography, vol. F* (Eds.: E. Arnold, F. Rossman), Kluwer Academic, Dordrecht, **2001**, pp. 333-351.

[3] R. O. Fox, F. M. Richards, *Nature* **1982**, *300*, 325-330.

[4] G. M. Sheldrick, SHELXL-97, Program for the Refinement of Crystal Structures, University of Göttingen, Göttingen (Germany), **1997**.

[5] G. Bunkóczi, L. Vértesy, G. M. Sheldrick, *Angew. Chem.* **2005**, *117*, 1364-1366; *Angew. Chem. Int. Ed.* **2005**, *44*, 1340-1342.

[6] M. Crisma, J. R. Deschamps, C. George, J. L. Flippen-Anderson, B. Kaptein, Q. B. Broxterman, A. Moretto, S. Oancea, M. Jost, F. Formaggio, C. Toniolo, *J. Pept. Res.* **2005**, *65*, 564-579.

[7] D. Cremer, J. A. Pople, *J. Am. Chem. Soc.* **1975**, *97*, 1354-1358.

[8] F. A. Allen, O. Kennard, D. G. Watson, L. Brammer, A. G. Orpen, R. Taylor, *J. Chem. Soc., Perkin Trans. II* **1987**, S1-S19.

[9] C. F. Snook, G. A. Woolley, G. Oliva, V. Pattabhi, S. F. Wood, T. L. Blundell, B. A. Wallace, *Structure* **1998**, *6***,** 783-792.

[10] G. Rispoli, *J. Photochem. Photobiol.* **1998**, *B44*, 1-20.

[11] A. Moriondo, G. Rispoli, *Photochem. Photobiol. Sci.* **2003**, *2*, 1292-1298.

[12] G. Rispoli, W.A. Sather, P.B. Detwiler, *J. Physiol.* **1993**, *465*, 513-537.

Table S1. Backbone torsion angles (°) in the [TOAC[16], Glu(OMe)[7,18,19]] alamethicin F50/5 analog

	Molecule A			Molecule B		
Residue*	ϕ	ψ	ω	ϕ	ψ	ω
Aib(1)	-46	-50	-176	-52	-36	-177
Pro(2)	-61	-42	179	-61	-29	176
Aib(3)	-58	-45	179	-52	-52	-178
Ala(4)	-63	-48	-178	-65	-46	-176
Aib(5)	-55	-48	-175	-58	-50	-178
Ala(6)	-71	-39	180	-71	-37	179
Glu(OMe)(7)	-62	-49	-175	-59	-41	-176
Aib(8)	-60	-45	-178	-57	-43	177
Val(9)	-63	-47	-177	-75	-46	-179
Aib(10)	-62	-39	180	-55	-45	-177
Gly(11)	-66	-37	-179	-66	-21	175
Leu(12)	-73	-26	-169	-73	-21	180
Aib(13)	-48	-49	-175	-55	-56	178
Pro(14)	-68	-24	-178	-65	-36	180
Val(15)	-65	-45	180	-56	-53	-178
TOAC(16)	-58	-53	-176	-57	-50	-177
Aib(17)	-54	-48	-174	-54	-48	-172
Glu(OMe)(18)	-69	-37	-173	-67	-36	-176
Glu(OMe)(19)	-88	-15	175	-86	-22	-176
Phol(20)	-141[†]	-57[‡]/ 49[§]		-120[†]	-71[‡]	

The estimated standard deviations are in the 0.7 ÷ 2.0° range.

*Residue numbering refers to molecule **A**. For molecule **B**, add 20.

[†]C19-N20-CA20-CT20 in molecule **A**, while C39-N40-CA40-CT40 in molecule **B**.

[‡]N20-CA20-CT20-OT20 in molecule **A**, while N40-CA40-CT40-OT40 in molecule **B**.

[§]N20-CA20-CT20-OT′20 (minor conformer).

Table S2. Side-chain torsion angles (°)

Residue	χ^1	χ^2	χ^3	§
Molecule **A**				
Glu(OMe)(7)	-179	-177	14, -177	-169
Val(9)	-68, 164			
Leu(12)	-151	30, 175		
Val(15)	-60, 176			
Glu(OMe)(18)	-70	162	-8, 179	175
Glu(OMe)(19)	-63	-179	1, 177	-179
Phol(20)	-160	80, -108		
Molecule **B**				
Glu(OMe)(27)	-77	174	2, -175	176
Val(29)	-66, 171			
Leu(32)	-72	-65, 172		
Val(35)	-61, 177			
Glu(OMe)(38)	-80	-161	-163, 16	-177
Glu(OMe)(39)	-67	179	-27, 155	179
Phol(40)	180	65, -112		

The estimated standard deviations are in the 0.7 ÷ 3.0° range.
The first of the two values of χ^3 for Glu(OMe) residues refers to the $\chi^{3,1}$ torsion angle involving the carbonyl oxygen OE1 atom.
The symbol § respresents the torsion angle CG-CD-OE2-CM.

Table S3. Intra- and intermolecular H-bond parameters

Type	Donor D-H	Acceptor A	Distance D … A (Å)	Distance H … A (Å)	Angle D-H … A (°)
Intramolecular, molecule **A**					
C_{10}	N3-H	O01	3.069	2.51	122
C_{13}	N4-H	O01	3.349	2.49	167
C_{13}	N5-H	O1	3.062	2.19	169
C_{13}	N6-H	O2	3.034	2.18	165
C_{13}	N7-H	O3	2.976	2.13	163
C_{13}	N8-H	O4	2.925	2.05	172
C_{13}	N9-H	O5	3.079	2.24	160
C_{13}	N10-H	O6	3.034	2.18	164
C_{13}	N11-H	O7	2.964	2.13	158
C_{13}	N12-H	O8	2.993	2.20	150
C_{13}	N13-H	O9	3.104	2.45	131
C_{10}	N15-H	O12	2.887	2.16	140
C_{13}	N17-H	O13	3.370	2.50	172
C_{13}	N18-H	O14	2.941	2.11	157
C_{13}	N19-H	O15	2.990	2.20	150
C_{13}	N20-H	O16	3.138	2.37	146
"oxy" C_{13}	OT20-HT	O17	2.783	2.15	132
"oxy" C_{13}	OT'20-HT'	O17	2.900	2.17	146
Intramolecular, molecule **B**					
C_{10}	N23-H	O021	2.816	2.10	137
C_{10}	N24-H	O21	3.011	2.48	120
C_{13}	N25-H	O21	3.128	2.26	169
C_{13}	N26-H	O22	2.876	2.01	168
C_{13}	N27-H	O23	2.992	2.17	155
C_{13}	N28-H	O24	3.069	2.25	156
C_{13}	N30-H	O26	2.935	2.06	172
C_{13}	N31-H	O27	2.778	1.92	165
C_{10}	N32-H	O29	2.923	2.23	136
C_{10}	N33-H	O30	3.076	2.28	150
C_{13}	N36-H	O32	2.793	1.92	172
C_{13}	N37-H	O33	3.082	2.22	166
C_{13}	N38-H	O34	2.919	2.10	155
C_{13}	N39-H	O35	3.048	2.25	151
C_{13}	N40-H	O36	2.915	2.19	139
"oxy" C_{13}	OT40-HT	O37	2.774	1.97	160
Intermolecular					
Peptide-water	N1-H	OW1	3.087	2.37	137
Peptide-peptide	N21-H	O38*	2.846	1.98	168
Water-water	OW1-HA	OW2	2.730	1.80	172
Water-nitroxide	OW1-HB	OD36[†]	3.060	2.21	179
Water-peptide	OW2-HA	O19[‡]	2.777	1.82	167
Water-peptide	OW2-HB	O25[§]	2.870	2.03	178
Water-peptide	OW3-HA	O10	2.608	1.78	168

*Symmetry equivalent x, y, -1+z. [†] Symmetry equivalent ½+x, -½+y, 1+z.
[‡]Symmetry equivalent x, y, 1+z. [§] Symmetry equivalent ½-x, -½+y, -z.