204. The Structure of the Polycyclic Nonadecapeptide *Ro 09-0198*

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In memoriam Prof. Dr. *Rolf Geiger*

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The conventional determination of the amino-acid sequence of the peptide antibiotic *Ro 09-0198* was prevented by four cyclizations *via* side chains. The joint application of NMR spectroscopy at highest field and automated *Edman* degradation yielded a complete determination of the connectivity pattern. The three-dimensional structure derived from NOE data exhibits an interesting separation of amino acids with hydrophilic and hydrophobic side chains.

Recently, we have presented the determination of the constitution of a polycyclic nonadecapeptide by two-dimensional ^{1}H-NMR spectroscopy [1]. This peptide, *Ro 09-0198*, belongs to the class of lanthionine- and methyllanthionine-containing polypeptide antibiotics which were named lantibiotics [2]. Sequencing and the determination of side-chain connectivities of these peptides is extremely difficult with conventional methods. Very recently, lantibiotics such as epidermin [3] were shown to be synthesized *via* prepeptides consisting of a leader sequence and the propeptide coded by their own structural gene [2].

Continuing the studies on the lantibiotic *Ro 09-0198*, we wanted to determine the conformation of this immunopotentiating peptide by evaluation of NOE's in combination with molecular-dynamics calculations [4]. However, first results showed that distance restraints derived from NOE build-up rates were incompatible with the previously proposed constitution (see fragment **A**) [1]. Therefore, it was necessary to check the proposed sequence. A prerequisite for the determination of sequential connectivities of amino acids in peptides and proteins by ^{1}H-NMR spectroscopy is the unequivocal assignment of all signals including the side-chain protons, especially when no other information of the peptide sequence is available. Due to severe overlap of signals even in 500-MHz spectra, misassignments could be the source of the above mentioned discrep-

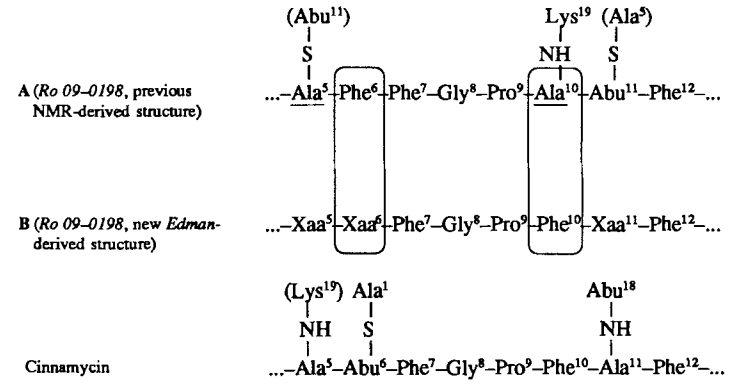

Fig. 1. *Peptide fragment of* Ro 09-0198 *as derived from NMR* (**A**) *and* Edman *degradation* (**B**) *and of the tentative sequence of the homologous peptide cinnamycin* [8]. Xaa resemble unknown amino-acid residues that are connected to other residues *via* their side chain. The underlined amino acids 5 and 11 in **A** have degenerate $H-C(2)$ signals. **A**: Ala^5-S-Abu^{11} and Abu^{11}-S-Ala^5 = Ala^5(3-Abu^{11}(4-SH)(S^4)) (β-methyllanthionine); Ala^{10}-NH-Lys^{19} = Ala^{10}(3-$Lys(N^6)$). **B**: no characterization of the amino acids Xaa. Cinnamycin: Abu^6-S-Ala^1 = Ala^1(3-Abu^6(4-SH)(S^4)) (β-methyllanthionine); Ala^{11}-S-Abu^{18} = Ala^{11}(3-Abu^{18}(4-SH)(S^4)) (β-methyllanthionine).

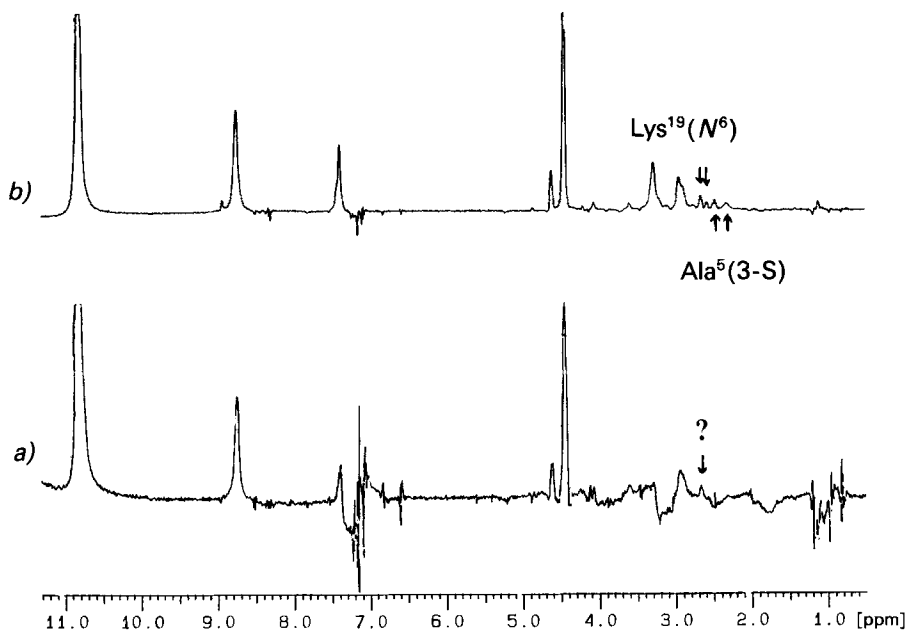

Fig. 2. *Two cross sections through the* NH *signal of the imino bridge in the* Ala^6*(3-*Lys^{19}*(*N^6*)) moiety from* a) *500-MHz- and* b) *600-MHz-NOESY spectra of* Ro 09-0198. Mixing time was 150 ms in both spectra. The cross peaks assigning the imino bridge of Ala^6(3-$Lys^{19}(N^6)$) are indicated by arrows and can easily be assigned in the 600-MHz spectrum, while they are hardly visible in the 500-MHz spectrum.

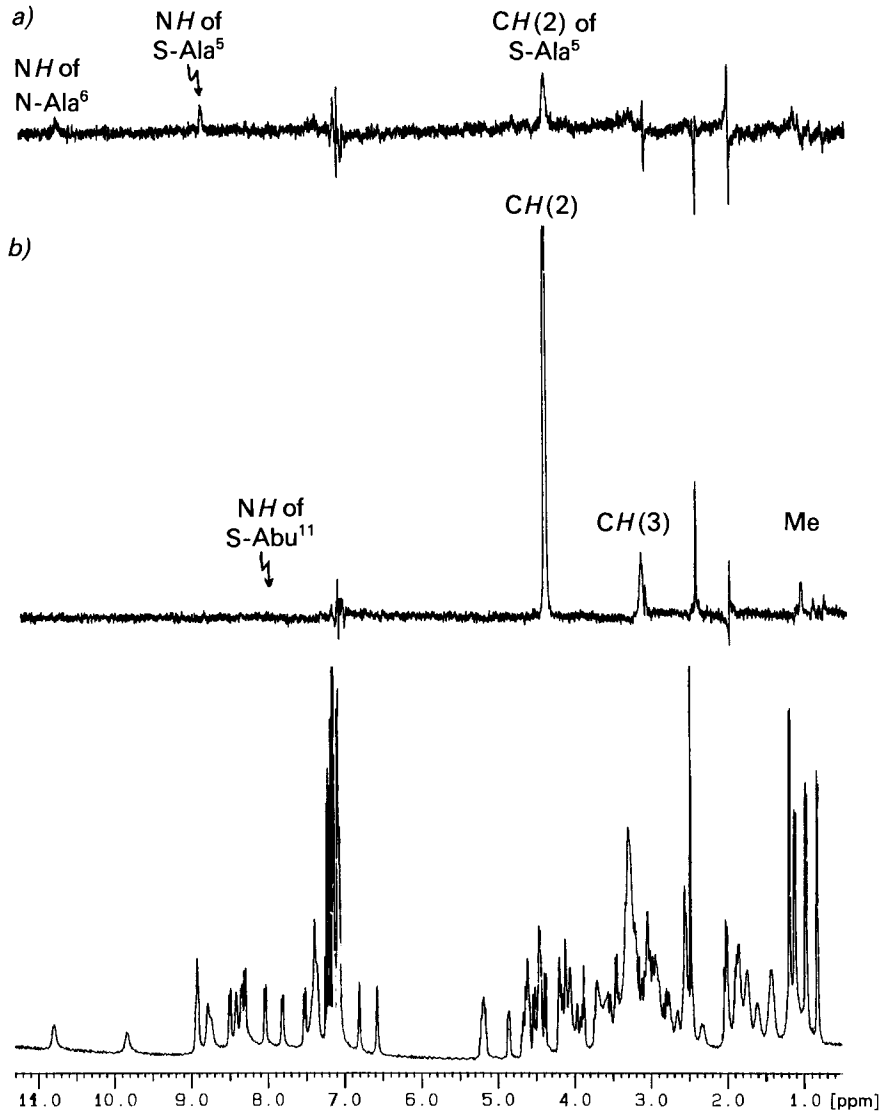

Fig. 3. *400-MHz 1D-RELAYED-NOESY of* Ro 09-0198[1]). The positions of the irradiated protons are marked by arrows. The excited magnetization is transferred *via J* coupling to C*H*(2), and the NOE's of the degenerate protons are observed in two separate experiments. The indicated NOE's confirm the originally proposed sequential arrangement of the spin systems (see text). No NOE of the C*H*(2) of S-Abu[11] can be seen because of the very broad N*H* signal of Phe[12]. No misassignments were made despite of the degeneracy of the two C*H*(2)'s. Not marked dispersive lines are remaining signals from incomplete subtraction.

[1]) Experimental parameters of the 1D-RELAYED-NOESY: Pulse-sequence: *D*1-*P*0-*D*2-*P*1-*D*3-*P*2-*D*3-*P*1-*D*4-*P*1-*AQ*; *D*1 = relaxation delay, 2.5 s; *P*0 = *Gaussian*-shaped frequency-selective pulse of 150 ms duration; *D*2 = 1/(2*J*) − *P*0/2 (in both spectra 2 μs); *D*3 = 1/(4*J*) for refocusing of antiphase magnetization (in a): 39 ms, according to 6.4 Hz; in b): 35.7 ms, according to 7 Hz); *D*4 = 180 ms and random variation within ±20 ms mixing time for NOE with suppression of *ZQC*; *P*1 and *P*2 are nonselective 90° and 180° pulses, resp.; phase cycling according to [10], measuring time 8 h each.

ancy. Hence, we carefully checked all assignments in an additional 600-MHz-NOESY spectrum [5], in which overlap is considerably reduced. We also performed a gas-phase sequence analysis to prove the positions of all *non-bridging* amino acids.

The use of automated *Edman* degradation for sequencing lantibiotics was demonstrated in the structural elucidation of pep5 [6] and gallidermin [7]. N-Terminal degradation of *Ro 09-0198* confirmed the NMR-derived amino-acid backbone, except at positions 6 and 10. At position 6, it could be shown that one moiety of a *bridging* amino acid must be located due to a gap found in the analysis of the phenylthiohydantoin derivatives. Furthermore, position 10 was clearly shown to consist of a phenylalanine. Hence, we suppose that the amino acids in positions 6 and 10 have been interchanged and that the fragment has the sequence **B** as shown in *Fig. 1*[2]).

The now found fragment **B** exhibits great similarities to the proposed sequence of the homologous peptide cinnamycin [8] in the primary peptide sequence. However, although the amino acids in the positions where bridging occurs are the same, the bridging itself is different to a large extend.

We now looked for further NMR evidence for the revised structure **B**. Phenylalanine (= Phe) and the alanine part of the lysinoalanine moiety (= Ala(3-Lys(N^6)) exhibit similar *AMXY* spin systems (*cf.* $C_6H_5CH_2CH(NH)CO$ and $OCCH(NH)(CH_2)_4NHCH_2CH(NH)CO$, resp.), because no scalar coupling of the imino proton of Ala(3-Lys(N^6)) is observed. The signals of the C*H*(3)'s of the two-spin systems are strongly overlapping at 3.00 ppm; NOE's of these protons with aromatic protons for Phe and CH_2(6) of Lys[19] for Ala(3-Lys(N^6)), respectively, have previously [1] been used to differentiate these two types of amino-acid moieties. However, they have been misassigned, interchanging their connectivities to the C*H*(2)'s. Evidence for the new assignment was now provided by *i)* a weak direct cross-peak in the 600-MHz NOESY spectrum between CH_2(6) of Lys[19] to C*H*(2) of residue 6 (Ala[6]) and *ii)* by an asymmetric NOESY-TOCSY peak [9] between the same protons indicating a two-step transfer $CH_2(6) \rightarrow CH_2(3) \rightarrow CH(2)$. Such a peak has not been found in the DQF-RELAYED-NOESY (because of its low intensity). Due to an additional severe overlap of the C*H*(2)'s, this interpretation had further to be checked: direct NOE cross peaks in the same 600-MHz NOESY spectrum from CH_2(6) of Lys[19] to the well separated N*H* of residue 6 *(Fig. 2)* give evidence for the revised structure.

To establish an unequivocal criterion for the sequential connectivity S-Ala[5]/N-Ala[6] (= Ala[5](3-Abu[11](4-SH)(S^4))/Ala[6](3-Lys[19](N^6))), an additional NMR experiment was needed, because the C*H*(2) of S-Ala[5] and the C*H*(2) of S-Abu[11] are overlapping. Hence, we recorded a 1D-RELAYED-NOESY spectrum [10] to enable a separated observation of the NOE's of the degenerate signals of the C*H*(2)'s of S-Ala[5] and S-Abu[11]. Starting from the selectively excited [11] magnetization of the isolated N*H*'s, this magnetization was transferred *via J* coupling to the corresponding C*H*(2)'s by means of a directed coherence transfer [10]. Subsequently, the NOE's of the individual C*H*(2)'s at degenerate chemical shift are observed. The results are shown in *Fig. 3*. Only the C*H*(2) of S-Ala[5] exhibits an NOE to the N*H* of N-Ala[6], while the C*H*(2) of S-Abu[11] does not, giving

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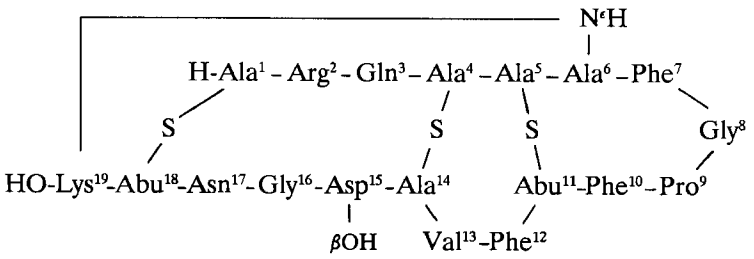
[2]) First presented at the Statusseminar 'Lanthionin-haltige Peptidantibiotika' in Tübingen, 11.12.1987 and the DFG-Symposium 'Nichtkovalente Wechselwirkungen' in Regensburg, 11./12.4.1988.



Ro 09-0198

Fig. 4. *Constitution of the polycyclic nonadecapeptide* Ro 09-0198. The presented amino-acid sequence results from *Edman* degradation in combination with gas-chromatographic detection of the phenylthiohydantoin derivatives of the non-bridging amino acids and new NMR investigations. Ala^{1}-S-Abu^{18} = Ala^{1}(3-Abu^{18}(4-SH)(-S^{4})), Ala^{4}-S-Ala^{14} = Ala^{4}(3-Ala^{14}(4-SH)(*S*^{4})), Ala^{5}-S-Abu^{11} = Ala^{5}(3-Abu^{11}(4-SH)(*S*^{4})), Ala^{6}-NH-Lys^{19} = Ala^{6}(3-Lys^{19}(*N*^{6})).

evidence that the *bridging* moiety in position 6 is not an Abu residue as proposed for cinnamycin.

The constitution resulting from the new investigations is given in *Fig. 4*[3]). It only differs from the original one in the exchange of the positions of a phenylalanine and of the alanine-part of the lysinoalanine moiety (*i.e.* Lys^{19}-HN$^{\varepsilon}$-Ala^{6} instead of Lys^{19}-HN$^{\varepsilon}$-Ala^{10} and Phe^{10} instead of Phe^{6}). Experimental distances obtained from quantified NOE's converge much better to a final structure using constraint molecular-dynamics calculations: For a total set of 228 distances, the mean deviation of the final structure is now 14 ppm. The thus obtained conformation exhibits an interesting feature: it turned out that hydrophilic and hydrophobic side chains are separated on opposite sites of the molecule *(Fig. 5)*. This would ideally lead to an orientation in the membrane similar to the one accepted for amphiphilic helices. The procedures leading to the conformation of *Ro 09-0198* will be described in a forthcoming paper.

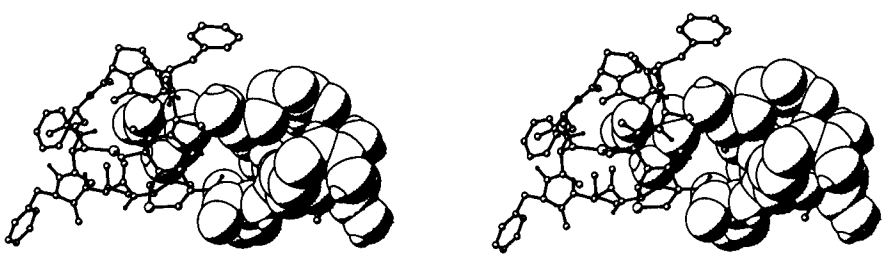


Fig. 5. *Conformation of* Ro 09-0198. The presented conformation is the result of constraint molecular dynamics calculations with a set of 228 distance restraints derived from quantified NOEs. Hydrophilic amino acids are presented by their *van der Waals* radii. They are spatially separated from hydrophobic residues.

[3]) **Note Added in Proof.** – After submission of our manuscript, an identical sequential result has been published: T. Wakamiya, K. Fukase, N. Naruse, M. Konishi, T. Shiba, *Tetrahedron Lett.* **1988**, *29*, 4771.

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