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Synthesis of Albicidin Derivatives: Assessing the Role of N-terminal Acylation on the Antibacterial Activity

Dennis Kerwat, Stefan Grätz, Julian Kretz, Maria Seidel, Maria Kunert, John B. Weston, and Roderich D. Süssmuth*[a]

The peptide antibiotic albicidin, which is synthesized by the plant pathogenic bacterium, *Xanthomonas albilineans,* represents the most prominent member of a new class of antibacterial gyrase inhibitors. It shows remarkable antibacterial activities against Gram-positive and Gram-negative microorganisms. Its unique structure potentially represents a new lead structure for the development of an antibacterial drug. Here we report the synthesis of 14 albicidin derivatives with structural variations at the N-terminus, primarily investigating the effects of variation of cinnamoyl, phenylpropanoyl, and benzoyl residues. Gyrase inhibition in vitro and determination of minimal inhibitory concentrations were assessed in parallel. Activities in a nanomolar range and the importance of N-acylation were demonstrated.

The development of antibiotic resistance by bacteria underlines the urgent need for new lead structures. Gram-negative bacteria, which have an outer membrane, are a particularly important group as this additional barrier makes it difficult to treat diseases caused by these bacteria.[1] A well-known group comprises *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeroginosa*, and *Enterobacter species*—the so called ESKAPE group.[2–4] They show a fast development of multidrug resistance, which leads to increasing problems to achieve successful patient treatment. One way to approach this problem is to search for natural products which show antibiotic activity. Many bacteria produce antimicrobial compounds as a survival technique to achieve an advantage over competitive microorganisms in their environment.[5–7] A new class of such compounds was recently discovered as represented by albicidin, but also by cystobactamide and coralmycin.[8,9] Albicidin has been isolated from the plant pathogen, *Xanthomonas albilineans*, which is the main agent causing leaf scald disease on sugar cane.[10,11] Leaf scald is important as it causes major crop losses every year.[12]

Albicidin was first described in the mid-1980s. Due to its low abundance in *X. albilineans* and its unusual oligo-aromatic character, it took more than 30 years to elucidate the structure.[13] It shows a strong antibacterial activity against Gram-positive and Gram-negative bacteria at nanomolar concentrations by acting primarily as a DNA gyrase inhibitor.[14–16]

Albicidin is an acyl pentapeptide composed of six building blocks, of which five are aromatic (Figure 1). The acyl unit on the N-terminus is a *para*-coumaric acid moiety featuring an additional α-methyl group (MCA, **A**). The pentapeptide consists of four δ-type aromatic amino acids. The two *para*-aminobenzoic acids (*p*ABA, **B** and **D**) are connected by an unusual amino acid β-L-cyanoalanine (L-Cya, **C**) to form the central tripeptide. The *C*-terminal dipeptide is composed of two identical substituted *p*ABA units, namely 4-amino-2-hydroxy-3-methoxybenzoic acid (*p*MBA, **E** and **F**). Recently some of our studies in structure elucidation revealed another naturally occurring albicidin with an O-carbamoylation on the *para*-coumaric acid. This modification enhances the activity to the lower nanomolar range.[17]

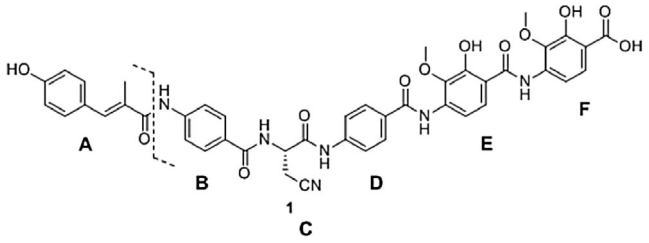

Figure 1. Structure of albicidin and its subdivision into the individual building blocks.

However, in spite of remarkable bioactivity there are resistance mechanisms already known, such as the resistance factor AlbD, which was recently investigated in our group.[18] As a consequence, the structure of albicidin needs to be optimized to withstand these resistance mechanisms.

The recently developed route towards a total synthesis of albicidin is based on the assembly of the molecule from six monomer building blocks which have been partially protected. The synthesis commences with the parallel assembly of the *C*-terminal dipeptide (**E**-**F**) starting from *ortho*-vanillin and of the central tripeptide (**B**-**C**-**D**) from, *p*-aminobenzoic acid and Boc-Asn-OH.[19] Subsequently these two fragments are combined to form a pentapeptide unit which after *N*-terminal deprotection undergoes coupling to introduce the *p*-hydroxy-2-methyl-cinnamoyl group. Global deprotection of the allyl-protecting groups affords albicidin in a total yield of 4.4%. As part of our continuing studies into the antibacterial effects of albicidins

[a] *D. Kerwat, S. Grätz, Dr. J. Kretz, M. Seidel, M. Kunert, Dr. J. B. Weston, Prof. Dr. R. D. Süssmuth*
Institut für Organische Chemie, Technische Universität Berlin
Straße des 17. Juni 124, 10623 Berlin (Germany)
E-mail: Suessmuth@chem.TU-Berlin.DE

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and investigation of the pharmacophoric regions of the molecule modulating or even being essential for the bioactivity, we focused on variation of the N-terminal acyl residue. Hence, we synthesized structural variants containing cinnamoyl, phenylpropanoyl, benzoyl, and acyl residues. This enabled assessment of the importance of the Michael system for bioactivity, as well as of steric and electronic requirements at the N-terminus. In summary it was found that a lipophilic, large acyl moiety is necessary to achieve proper antimicrobial activity.

Following the established synthesis protocol, we were able to provide the subfragment **2** (Figure 2) as a precursor to albicidin analogs in a multigram scale. In addition to the naturally occurring substituted cinnamic acid found in albicidin and close derivatives, we were able to couple a range of acids, including phenylpropanoyl acids, benzoic acids, and fatty acids.

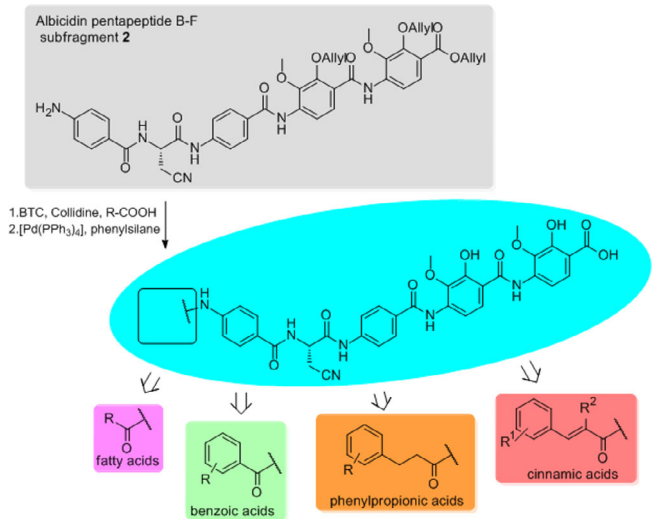

Figure 2. Combinatorial approach for new albicidin derivatives. Several sets of carboxylic acids coupled onto the subfragment **2**.

The penultimate step in the established total synthesis strategy for albicidin comprises the coupling of the N-terminal cinnamoyl residue. Optimized synthesis enabled synthesis of multigram quantities of subfragment **2** (Figure 2), and therefore it was chosen as key intermediate for performing the study of N-terminal variations. We selected four sets of acyl residues to assess their influence on gyrase inhibition and antibacterial activity: The first set of acid building blocks comprised various cinnamic acids (Figure 3a) which were chosen to establish the importance of steric and electronic factors as well as the effects of substituting the cinnamoyl double bond. Albicidin derivatives **3** and **4** assess the importance of the OH group in the, *para*-position in comparison to *m*-OH and *o*-OH analogues. Then albicidins **5–9** represent the absence of an aromatic substituent (**5**), substitution with fluorine (**6**), carboxylate (**7**), amino (**8**), and trifluoromethyl (**9**) groups. Finally, derivatives **10** and **11** explore the influence of the methyl group on the alkene in the presence and the absence of the 4-hydroxy substituent. The synthesis of the commercially unavailable cinnamic acids in high yields was quite straightforward according to previously published protocols[20] starting from the correspondingly substituted benzaldehyde and methylmalonic acid (see also Supporting Information). If required, functional groups were protected as allyloxy or Alloc-amino and then coupled to subfragment **2** in yields of 70–98%. Global deprotection was performed using tetrakis(triphenylphosphine)palladium(0) as the catalyst and phenylsilane as the scavenger to give moderate yields of about 30% of target compounds. The final purification was carried out via reversed phase high-performance liquid chromatography (HPLC). The purity of the albicidin derivatives was determined by HPLC-MS and NMR spectroscopy (Supporting Information).

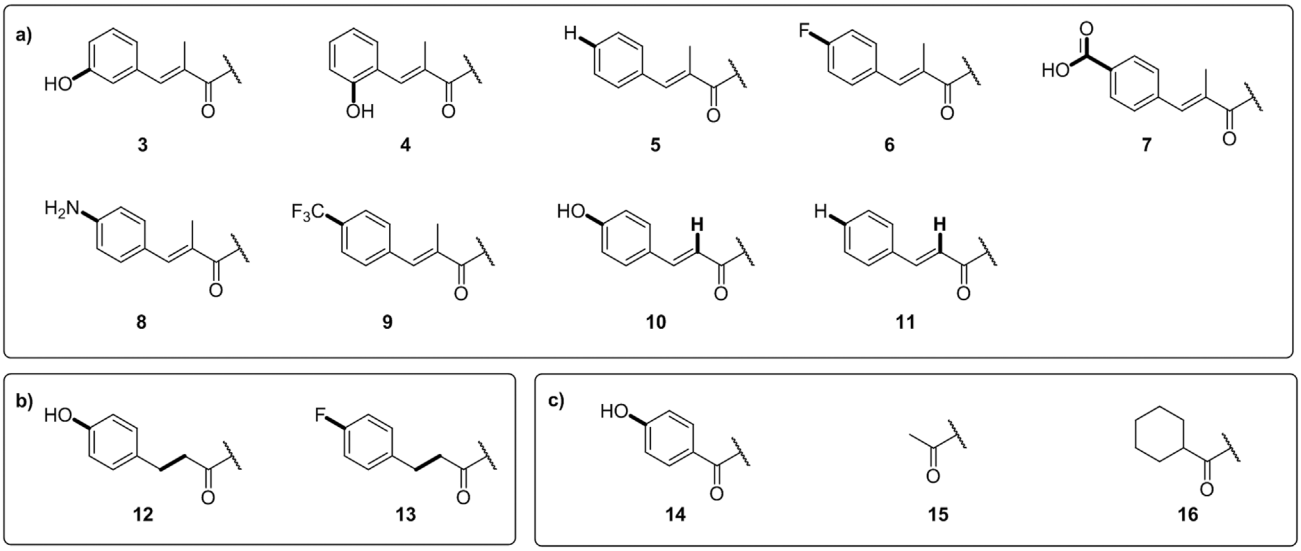

Figure 3. Overview of the building block **A** variations. a) Variation of substitutions on the cinnamoyl residue including variation of the position of the –OH group. b) Phenylpropanoyl residues without a Michael system. c) Short length benzoyl and acyl residues.

The second set comprised phenylpropanoyl derivatives (compounds **12** and **13**, Figure 3b) which on the one hand probe the significance of the Michael system for bioactivity, and on the other hand flexibility. These derivatives, formed by insertion of an ethane linker consisting of sp^3 carbons, are conformationally flexible between the subfragment structure and the N-terminal aromatic ring. It has been known for a long time that cinnamic acids tend to cyclo-dimerize when exposed to UV radiation.[21] Thus it seemed worthwhile to make these analogues without the Michael system to avoid the possibility of dimerization, as this might be expected to cause a loss of antimicrobial activity. In derivative **14**, which bears a benzoyl group, the linking region has been deleted (Figure 3c). Synthesis, coupling, and deprotection have been carried out under the same conditions as described for albicidin for the above set of cinnamic acids.

Finally, acetylation (**15**) as well as replacement of the aromatic ring with a cyclohexyl-substituent (**16**) were investigated (Figure 3c). We aimed to decrease the molecular weight (MW) of the target derivatives as much as possible. Since albicidin is a natural product that is a member of one of the orally active therapeutic classes which are antibiotics, antifungals, vitamins, and cardiac glycosides, Lipinski's "rule of 5" does not strictly apply.[22] However, this provides a useful guideline to work from, and when molecular weights become too large, this lowers the "drug-likeness" of the compound. Comparing albicidin and its derivatives with Lipinski's "rule of 5" results in an outnumbering of each component of the rule. Of course the number of hydrogen bond donors (HBDs), hydrogen bond acceptors (HBAs), and the MW differ from each compound, but the MW ranges from 724 (**15**) to 984 (**9**) Da and is in every case clearly above 500 Da. The maximum number of HBDs, which should be 5, as well as the HBAs (ideally 10) is clearly exceeded (8 to 9 HBDs, respectively 15 to 17 HBAs).

DNA gyrase is the primary molecular target for albicidins. The new compounds were therefore assessed for their inhibition of DNA gyrase from *E. coli*. The half maximal inhibitory concentration (IC_{50}) value of albicidin was determined to be approximately 40 nM.[19] The assay was then carried out at this concentration for all newly synthesized compounds and the results are shown in Figure 4.

Initially, the location of the OH-group on the cinnamoyl residue was investigated. The naturally occurring albicidin has OH in the *para*-position. Derivatives with *m*-phenol (**3**) and *o*-phenol (**4**) and also lacking a hydroxy-group (**5**) were synthesized. The data in Figure 4a indicate that these derivatives are virtually devoid of inhibition of gyrase supercoiling at a concentration of 40 nM. Therefore we investigated several alternative substituents in the *para*-position. Initial studies for albicidin reveal a moderate microsomal instability (unpublished data) and to enhance metabolic stability, we replaced the hydroxy group with a fluorine (**6**). Increasing lipophilicity can enhance membrane permeability, and this can lead to a better accessibility to the target. For this reason we also made the analogue with trifluoromethyl in the *para*-position (**9**), and this showed similar activity to albicidin. Along with their lipophilicity, the electron withdrawing effects of compound **6** and **9** could also

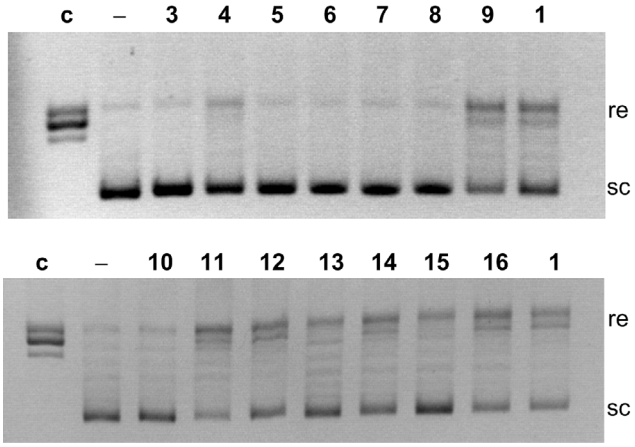

Figure 4. Inhibition assay of, *E. coli* DNA gyrase supercoiling activity for the novel albicidins prepared. The control experiment without enzyme and drug (lane c) shows relaxed DNA (re) and the addition of enzyme (lane –) results in supercoiled DNA (sc). The other lanes represent the reaction with enzyme and derivatives. a) Derivatives **3**–**9**, with albicidin (**1**) in the right lane. b) Derivatives **10**–**16** with albicidin (**1**) in the right lane. The assay was carried out at the IC_{50} of albicidin (40 nM). Values obtained from two independent experiments.

be important for the activity. We then investigated the role of molecular weight, in particular the decrease of MW. Initially we synthesized the analogue **10** without a methyl substituent on the double bond, and it revealed less activity than albicidin. Interestingly compound **11** (no *p*-OH and no methyl on the double bond) was similar in activity to albicidin, whilst having a reduced molecular weight. But since it is only a loss of 4% of weight, it may not affect the permeability and the solubility properties. To decrease the molecular weight further, we coupled benzoic acids and fatty acids to the subfragment **2**. The resulting benzoyl compound **14** showed a similar activity profile. Encouragingly, it was the most soluble compound among those prepared, based upon estimations from the retention time in HR-LCMS (gradient details available in the Supporting Information). The retention times on HPLC systems give a good indication of the lipophilicity. But since the solubility issue is a more complex one, we are measuring the solubility in a separate experiment (unpublished data). The smallest coupled residue was the acetyl group that led to derivative **15** which was only a weak inhibitor of the gyrase. Attaching the cyclohexylcarboxylic acid (**16**) retained the activity of albicidin but decreased the solubility of the compound.

We also investigated the importance of the Michael system, as mentioned above, because it is known that cinnamic acids tend to dimerize when exposed to UV light.[21] We synthesized the saturated analogues **12** and **13** which contain phenylpropanoyl acids with a hydroxy group and a fluorine in the, *para*-position respectively. Both compounds were less active than their cinnamoyl counterparts but revealed a similar trend, that fluorine in the *para*-position increases the potency.

Finally, the antimicrobial activities for all compounds were determined against a set of Gram-positive and Gram-negative bacteria (Table 1). When we compared the obtained data from the gyrase assay with the results from the cell-based assay we

Table 1. Antibacterial activity of albicidin and synthetic derivatives

Compound	*B. subtilis* DSM10	*M. Luteus* DSM1790	*S. typhimurium TA100*	*E. coli* DSM1116
Albicidin	297	2375	37	74
3	1187	19000	148	297
4	2375	19000	148	594
5	605	2432	76	151
6	296	1185	19	73
7	>9000	>18000	1150	9195
8	1192	596	297	594
9	280	1120	70	140
10	600	4832	151	151
11	616	4926	154	154
12	4819	>19000	602	2401
13	600	9615	150	600
14	4987	>20000	156	623
15	11000	>22000	>11000	>11000
16	10000	20000	630	10000

[a] Minimum inhibitory concentration (MIC) values are given in nM.[a]For experimental details, see the Supporting Information. Values were obtained from two replicates. If deviation occurred, the upper magnitude is shown in the Table.

could observe a general trend between the two, though some inconsistencies in the activities could be observed.

These inconsistencies could arise due to many effects. The *E. coli* DNA gyrase assay was conducted at a fixed concentration of 40 nM for each compound, matching the IC_{50} value of albicidin. This allowed us to compare the activity of all derivatives directly with one to another. In the upper picture in Figure 4 the compounds **3**–**8**, which showed activity in the cell-based assay, seem to be inactive towards the *E. coli* gyrase. In fact, they are active, but they have a higher IC_{50} value. Since we are searching for potential gyrase inhibitors, with comparable or even higher activity towards the gyrase than albicidin, the IC_{50} determination was not performed for compounds with $IC_{50} > 40$ nM. However, as an example, the IC_{50} value of compound 8 was determined to be 230 nM (see Supporting Information).

In the cell-based assay we tested with a range of concentrations for each compound. In this assay, other parameters come into play. The activity depends on the cellular uptake and the resulting effective intracellular concentration of compounds. Thus, the concentration directly accessible to the target enzyme may differ from the applied concentration in the assay due to various mechanisms. Intracellular concentrations can depend on the membrane permeability, which could be influenced by the different acyl moieties. In addition, some derivatives may be digested proteolytically faster than others. As we mentioned before there are already known resistance mechanisms against albicidin. One of these mechanisms concerns efflux pumps which are known to actively lower the intracellular concentration of drugs and could, in this case, lower the concentration of the albicidin analogues.[23,24] As an example, the derivatives **12**, **14**, and **16** retained activity in the target-directed assay at a similar level to that for albicidin, but the compounds were less potent in the cell-based assay. In this case, increased lipophilicity apparently did not lead to enhanced membrane permeability. We will investigate the properties of the albicidins with respect to membrane permeability and proteolytic stability and will further probe the known resistance mechanisms. Additionally, the *E. coli* DNA gyrase assay may only be one indicator of the antibacterial activity of albicidin, as there have been reports of albicidin's activity towards other enzymes, such as TopoIV,[25] which may be responsible for the observed inhibitory activity, where poor gyrase inhibition is observed for example, compound **6**.

In summary, we carried out the synthesis of 14 new albicidin derivatives and have afforded the first insights into the structure–activity relationship of these compounds when considering the variations of the N-terminal acyl group. Based on this limited series of compounds, it seems that considerable variation of structure of the N-terminus with retention of activity is permitted. Substitution in the *para*-position of the benzoyl moieties may be important for activity. It appears that the total length of the molecule is also important for the activity since the N-acetylated derivative **15** was almost inactive. Thus, we synthesized compounds with nanomolar activity against DNA gyrase and potent antibacterial activity which demonstrates the potential of albicidin analogues to generate novel antibacterial drugs.

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Keywords: albicidin · antibacterials · antibiotics · gyrase · *para*-aminobenzoic acid · peptides

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*D. Kerwat, S. Grätz, J. Kretz, M. Seidel, M. Kunert, J. B. Weston, R. D. Süssmuth**

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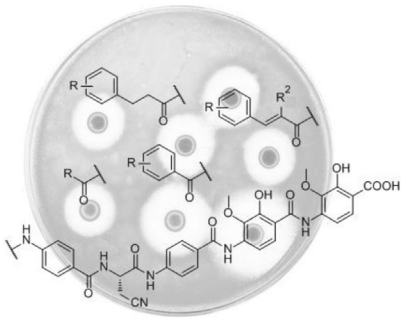
Synthesis of Albicidin Derivatives: Assessing the Role ofN-terminal Acylation on the Antibacterial Activity



Advantages of acylated albicidin: The peptide antibiotic albicidin represents the most prominent member of a new class of antibacterial gyrase inhibitors. It shows remarkable antibacterial activities against Gram-positive and Gram-negative microorganisms. 14 newly synthesized albicidin derivatives with structural variations at the N-terminus are reported here. Gyrase inhibition and determination of minimal inhibitorial concentrations were assessed in parallel to show activities in a nM range and the necessity of N-acylation.