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Received: April 3, 2003 [Z617]

Aminoacyl Adenylate Substrate Analogues for the Inhibition of Adenylation Domains of Nonribosomal Peptide Synthetases

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KEYWORDS:

adenylation domain · enzymes · inhibitors · nonribosomal peptide synthetases · synthetases

Microorganisms are well-known producers of bioactive peptides, which have become important elements of modern medicine. The sphere of activity of these compounds is vast and ranges from antibiotic to immunosuppressive, cytostatic to toxic effects. For instance, among these peptides are important antimicrobial agents such as the heptapeptide vancomycin, and immunosuppressive substances such as the cyclic undecapeptide cyclosporine.[1, 2] Both of these compounds are produced by nonribosomal peptide synthetases (NRPS), which are complex enzymes organized in modules that catalyze multistep reactions.[3, 4] A module needed for the incorporation of one amino acid into the final product comprises an adenylation (A) domain for substrate recognition, a 4′-phosphopantetheine-dependent carrier protein that holds the activated substrate and the growing chain, and a condensation (C) domain for peptide bond formation. Additionally, the activated substrate may also be altered by NRPS-associated optional domains that epimerize, methylate, or *N*-formylate, or by postsynthetic enzymes that glycosylate or halogenate the substrate; these alterations make the final products not easily accessible through chemical synthesis.[5] A domains activate, in addition to proteinogenic amino acids, a number of nonproteinogenic amino acids as well as carboxy acid. These domains specifically activate one substrate as the corresponding acyl adenylate at the expense of adenosine triphosphate (ATP).[6–8] Two crystal structures of A domains have been solved. The crystal structure of the gramicidin S-synthetase A (GrsA) phenylalanine-activating A domain from *Bacillus brevis* led to the elucidation of the so-called "nonribosomal code," which allows the prediction of the specificity of an A domain.[9, 10] This prediction was limited to amino-acid-activating A domains until the structure of dihydroxybenzoate-adenosine monophosphate ligase (DhbE) was solved recently, which further extended the code to include carboxy-acid-activating A domains.[11] Although the NRPS A domains exhibit the same enzymatic activity as other adenylate-forming enzymes, such as aminoacyl tRNA synthetases of ribosomal protein synthesis, these enzymes are not related on either the sequence or the structural level.[12–15] Instead, A domains are structurally related to the luciferase from *Photinus pyralis*.[16] We present herein the application of the first A domain inhibitors. Not only did these acyl adenylate analogues prove to be excellent inhibitors, their modification with linkers, which allows binding to a matrix, enlarges the number of possible applications.

Aminoacyl tRNA synthetases have successfully been inhibited by the aminoacyl analogues 5′-*O*-[*N*-(aminoacyl)-sulfamoyl] adenosine.[17] For instance, the alanyl-tRNA-synthetase from *Escherichia coli* can be inhibited by the alanyl derivative of this compound.[18] Following this approach, we synthesized the corresponding 5′-*O*-[*N*-(phenylalanyl)-sulfamoyl] adenosine (**1**) and 5′-*O*-[*N*-(leucyl)-sulfamoyl] adenosine (**2**) to test whether these analogues also inhibit A domains based on the similarity in enzymatic activity of such domains. Two A domains were chosen to test the functionality of the analogues **1** and **2**. We chose the GrsA A domain (PheA) because it has been crystallized in the presence of the substrates adenosine monophosphate and phenylalanine, which yielded a detailed insight into the mechanism of binding and catalysis of this domain. The second A domain, LeuA, is the excised A domain of the surfactin synthetase C (SrfA-C) from *Bacillus subtilis*. The corresponding gene fragments of the A domains, namely *PheA* and *LeuA*, were cloned (Figure 1) and the proteins were produced as strep-tag II fusions in *E. coli* and subsequently purified by streptactin affinity chromatography.

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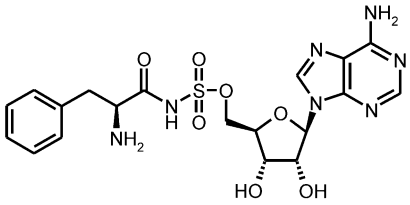
 DOI: 10.1002/cbic.200300666 © 2003 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



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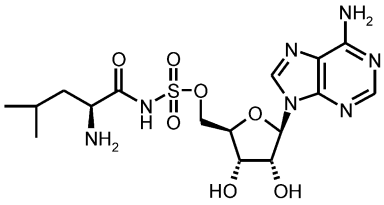

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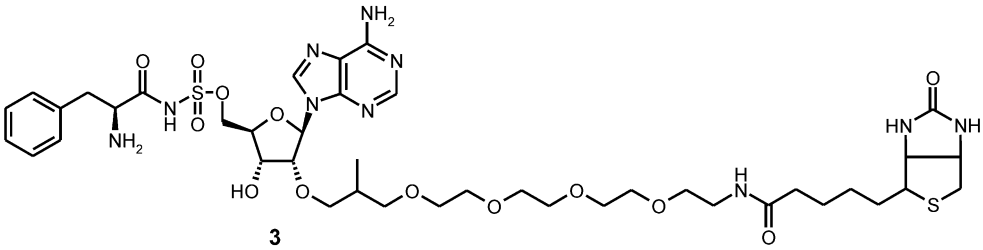

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Before the conduction of any further experiments, the substrate specificity and kinetic constants of both enzymes were determined. PheA specifically activated L- and D-phenylalanine. LeuA activated L-leucine with a slight side-specificity for L-isoleucine, as expected (not shown). To allow better comparison with the inhibition studies to follow, Michaelis constants K_m of both PheA and LeuA for their respective substrate amino acid were determined by ATP/inorganic pyrophosphate (PP_i) exchange assay. This assay measures the back reaction of the adenylate formation. The use of ^{32}P-pyrophosphate in these assays leads to incorporation of ^{32}P-phosphate into ATP in direct proportion to the activity of an A domain. For this purpose, the enzymes were incubated with either L- or D-Phe in the case of PheA, or L-Leu in the case of LeuA. PheA exhibited K_m values of 9.3 ± 0.8 μM and 19.0 ± 1.3 μM for L-Phe and D-Phe, respectively. LeuA had a K_m value of 642 ± 36 μM for L-Leu. The values for PheA differ significantly from those previously determined for the same protein when His-tagged.[7] Differences in protein-tagging, purification strategy, as well as buffer system may account for this increased K_m value. Values for LeuA are in good agreement with those formerly measured.[19]

Before the determination of quantitative data for both enzymes with their respective inhibitor, a qualitative assay was used to determine the quality and specificity of inhibition by **1** and **2**. These and later assays with a linker-modified inhibitor (see below) showed inhibition resulting in up to total loss of activity of the A domains. In addition, no cross inhibition was observed between the two A domains, which indicates the expected specificity of the inhibitors (not shown). For the determination of inhibition constants, K_i, the concentration of the inhibitor was varied while the substrate concentration was kept constant. Three different sets of data points were collected for each of the two A domains. The concentration of the inhibitor was plotted against the reciprocal of the reaction rate in Dixon plots produced using results from the ATP/PP_i exchange assays. The two K_i values found for 5′-*O*-[*N*-(L-phenyl)-sulfamoyl] adenosine (**1**) with PheA and either L-Phe or D-Phe as substrate are the same (within experimental error), at 61 nM and 63 nM, respectively (Figure 2a and b). The K_i value for 5′-*O*-[*N*-(L-leucyl)-sulfamoyl] adenosine (**2**) with LeuA was even lower at 8.4 nM (Figure 2c). These values, which are up to five orders of magnitude lower than the respective K_m values of the A domains, make these compounds especially suitable as inhibitors. It is surprising that, despite the fact that the conformation of the adenylate formed by A domains differs a great deal from that formed by tRNA synthetases, the same inhibitors can be used for both classes of

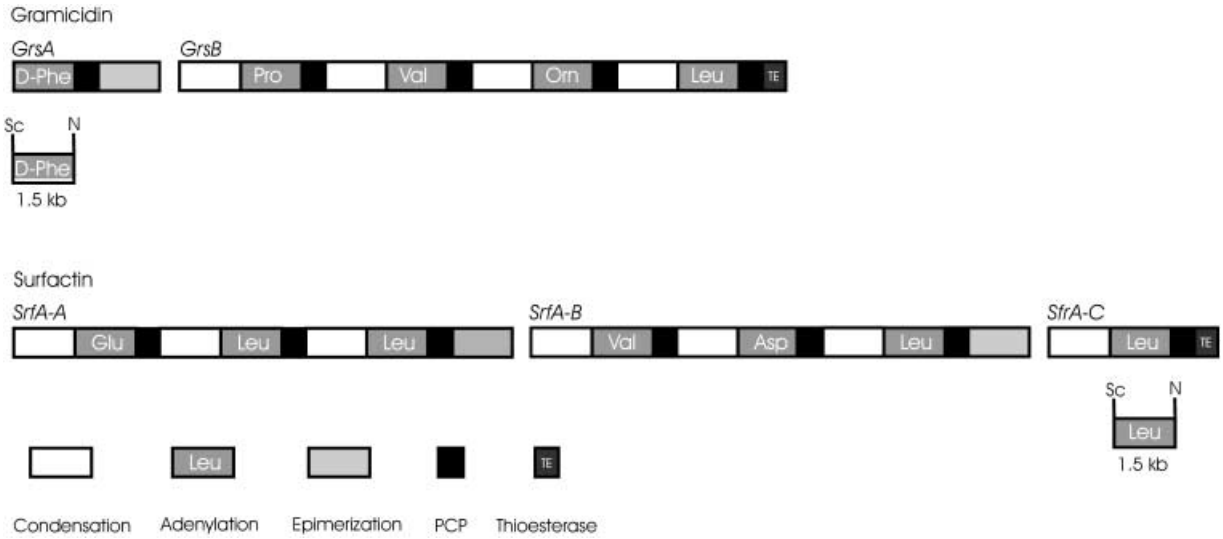

Figure 1. Modular organization of the gramicidin S and surfactin biosynthesis operons showing the exact position of the A domains chosen for cloning. Sc, SacII; N, NcoI, PCP, peptidyl carrier protein domain.

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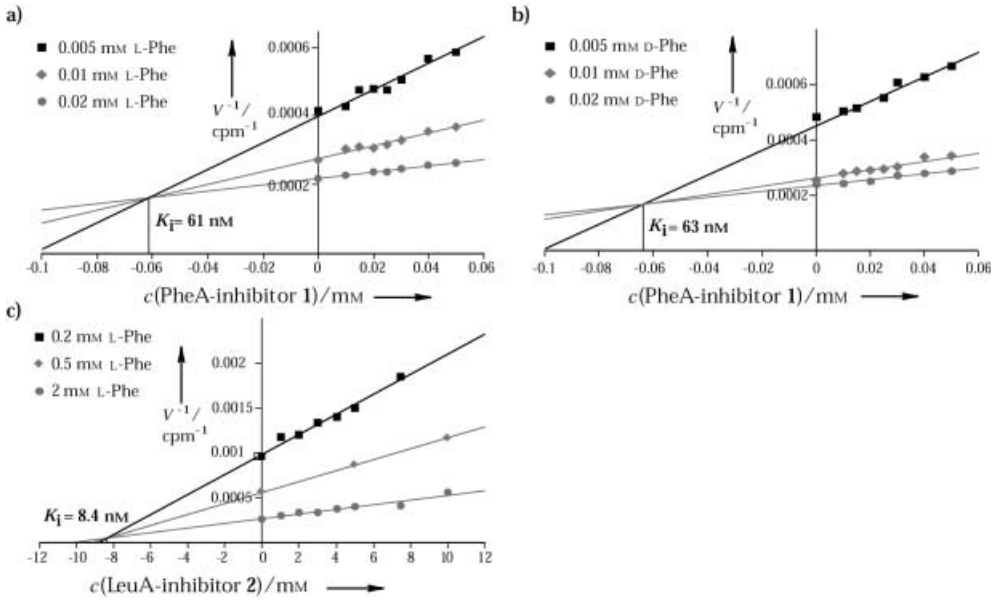

Figure 2. *Dixon plots of inhibition studies with the recombinant proteins and the linkerless inhibitors **1** and **2**. A) PheA with inhibitor **1** and L-Phe. B) PheA with inhibitor **1** and D-Phe. C) LeuA with inhibitor **2** and L-Leu. The concentration of the inhibitors was varied as follows: PheA, 0 – 0.05 μM; LeuA, 0 – 10 nM. Amino acid concentrations were as indicated in the figure. cpm, counts per minute.*

enzyme.[15] Moreover, the kinetic and inhibitory constants for PheA are comparable to those determined for the isoleucyl-tRNA synthetase from *Staphylococcus aureus*.[20, 21]

Comparison of the structure of the GrsA A domain with bound substrate with the luciferase structure without substrate indicated closing of the active site when the substrate is bound, as was shown for the lysyl tRNA synthetase from *Thermus thermophilus* in the presence of a sulfamoyl analogue of lysyl adenylate.[9, 22] Thus, such inhibitors may be used to study the reaction mechanism of NRPS A domains and may also facilitate their crystallization. Crystallization of whole NRPS modules has failed to date and we anticipate that these inhibitors may be of value for freezing the otherwise flexible structure of such modules in one state and thus making crystallization possible. In addition, these inhibitors may also open the door to chemical genetics, that is, the use of nonproteinogenic aminoacyl-sulfamoyl-adenosine derivatives to pre-terminate or inhibit NRPS pathways.[23] Also, to explore the possibility of using these adenylate analogues as matrix-bound inhibitors, we synthesized a phenyl adenylate analogue with a flexible 2′-*O*-[polyether-biotin] linker[24] (Compound **3**). The biotin serves as the site for attachment to a streptactin or streptavidin matrix. We tested this linker-modified inhibitor for functionality and found that it shows no difference in inhibition compared to the linkerless inhibitor. As can be seen from Figure 3, equal amounts of the linkerless inhibitor **1** and linker-modified inhibitor **3** lead to equal inhibition, which indicates that both inhibitors have the same K_i value.

By using a method similar to that described by Keefe et al., A domains with changed specificity could be isolated from a library of A domains created by error-prone PCR followed by production of the enzymes in vitro.[25] Thus, these inhibitors represent powerful tools to alter NRPSs with the aim of creating peptides with novel activities.

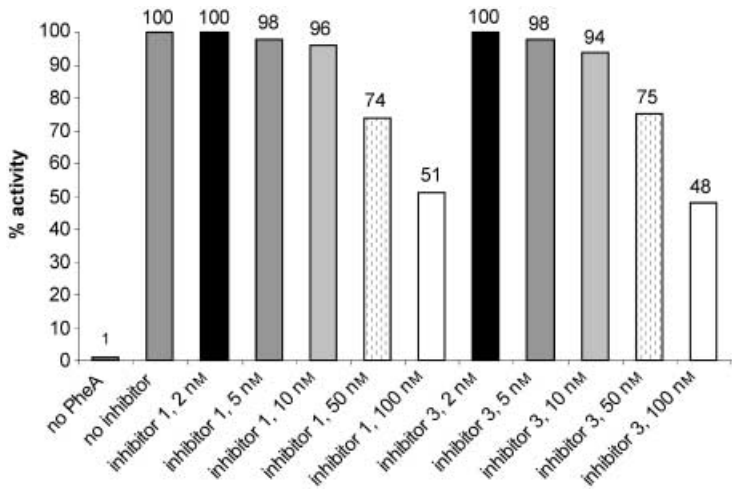

Figure 3. *Comparative study of the linkerless inhibitor **1** and the linker-modified inhibitor **3**. The concentration of both inhibitors was varied from 2 – 100 nM while the amino acid concentration was kept constant in ATP/PP$_i$ exchange assays. Corresponding columns have the same grey scale.*

These examples illustrate the generality of this approach to generating selective inhibitors for NRPS A domains. Although several hundred different substrates of A domains have been uncovered to date, minor changes in the synthesis should suffice to produce the desired specific inhibitor for any A domain.[4]

Work in MAM's laboratory was supported by the Deutsche Forschungsgemeinschaft, Fonds der Chemischen Industrie, and Aventis Research & Technologies.

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Received: May 20, 2003 [Z 666]

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