Amino Acyl-Adenylate Substrate Analogues for the Inhibition of Adenylation Domains of Nonribosomal Peptide Synthetases

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Synthesis of inhibitors

The aminoacyl sulfamates 1 and 2 were prepared from the known precursor 4 analogously as described in the literature (scheme 1). The inhibitor 3 was prepared in about 20 steps by procedures that will be described elsewhere in due course.

Preparation of 5'-O-[N-(L-phenylalanyl)-sulfamoyl]-adenosine (1)

Scheme 1: Synthesis of 5'-O-[N-(L-phenyl)-sulfamoyl] adenosine

2',3'-O-Isopropylidene-5'-O-sulfamoyladenosine 4 (1.4 g, 3.6 mmol) was dissolved in N,N-dimethylformamide (DMF, 22 mL). After addition of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 550 mg, 3.6 mmol), N-Boc-L-phenylalanine-O-succinimide ester 5 (1.3 g, 3.6 mmol) was added in portions within 50 min. After stirring for 3 h at room temperature, the mixture was concentrated and the residue was taken up in water and extracted with dichloromethane. The
organic layer was dried over Na$_2$SO$_4$, concentrated and purified via chromatography on silica gel by elution with chloroform/methanol 7/1 to yield the 2', 3'-O-isopropylidene protected compound 6 as a colorless solid (1.3 g, yield 57%) which was directly transformed into 1 by the following procedure.

Compound 6 (0.90 g, 1.42 mmol) was dissolved in a mixture of trifluoroacetic acid (TFA) and water (14 ml, 5:2 v/v) and stirred for 5 h at room temperature. The reaction mixture was concentrated in vacuo and co-evaporated four times with ethanol. The residue was suspended in ethanol. The solid was filtered off, washed and dried to give slightly yellow crystals of 1 (111 mg, yield 18%). The filtrate was concentrated and subjected to the reaction conditions to yield further 188 mg of product.

**Preparation of 5'-O-[N-(L-leucinyl)-sulfamoyl]-adenosine (2)**

This inhibitor was obtained by following the above described procedure from 4 and the corresponding N-Boc-L-leucine-0-succinimide ester via the 2',3'-O-isopropylidene protected intermediate (yield 73%) and deprotection (yield 24%).

**HPLC purification of the inhibitors 1 and 2**

HPLC purifications were carried out on the Vision Workstation, BioCAD Family (PerSeptiveBiosystems) on a LiChroCART 250-10 column, LiChrosspher 100, RP18 10 µM (Merck, Darmstadt, Germany). The following conditions were used:
Flow rate: 3 mL/min
UV-detector: 260 and 275 nm
Equilibration block: 100% water for 10 min
Load block: Injection volume up to 500 µL
Wash block: 100% water for 2 min
Elute block: gradient to 80% acetonitrile in 100 min
Clean block: 100% acetonitrile for 5 min

Microbiology and Biochemistry

General techniques

E. coli was grown on LB medium. Antibiotics were used at the following concentrations, ampicillin 100 µg/mL. For E. coli techniques, such as transformation and plasmid preparation, standard protocols were used. Vent polymerase (New England Biolabs, Schwalbach, Germany) or Pwo polymerase (Roche, Mannheim, Germany) was used to amplify gene fragments for cloning and expression purposes. Oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany).

Construction of plasmids

Construction of pASK-IBA3-PheA - A fragment of the grsA gene encoding the B. brevis GrsA-A-domain (PheA) was amplified by PCR with oligonucleotides 5'-TATCCGCCTCATGCTCAAAATAAAAATGG-3' and 5'-TATCCATGGGTTAAATCAGGGTCCGGC-3' (restriction sites are underlined) from chromosomal DNA of B. brevis ATCC 9999 and, after restriction digest of the amplified fragment, ligated into the SacII and NcoI sites of pASK-IBA3 (IBA, Göttingen, Germany). The resulting plasmid pASK-IBA3-PheA encodes the recombinant PheA with a C-terminal tag GLSAWSPQFEK and four additional, N-terminal amino acids, GDRG.
Construction of pASK-IBA3-LeuA - A fragment of the srfA-C gene encoding the B. subtilis SrfA-C-A-domain (LeuA) was amplified by PCR with oligonucleotides 5'-TATCCGCGGGCCTGTCAGCACGATCAA-3' and 5'-TATCCATGGTCCTGATCAGGTTTTGGGAG-3' from chromosomal DNA of B. subtilis ATCC 21332 and, after restriction digest of the amplified fragment, ligated into the SacII and NcoI sites of pASK-IBA3 (IBA, Göttingen, Germany). The resulting plasmid pASK-IBA3-LeuA encodes the recombinant LeuA with a C-terminal tag GLSAWSHPQFEK and four additional, N-terminal amino acids, GDRG.

Overproduction and purification of recombinant proteins

E. coli XL1-blue (Stratagene, Heidelberg, Germany) was transformed with pASK-IBA3-PheA or pASK-IBA3-LeuA, for the production of the Strep-Tag II fusion proteins PheA and LeuA. 1 mL of an overnight culture of these strains in LB was inoculated into 100 mL of the same medium. The culture was grown at 30 °C and 250 rpm. Expression was induced by addition of 5 µL of an anhydrotetracyclin solution (2 mg/mL DMF) at an A₆₀₀ of 0.5 and the culture was allowed to grow for an additional 3 h before being harvested by centrifugation at 4,500 g and 4°C. The cells were resuspended in 1.5 mL buffer W (100 mM Tris/HCl, 1 mM EDTA (pH 8.0)), and disrupted by three passages through a cooled French pressure cell. The resulting crude extract was centrifuged at 36,000 g at 4°C for 30 min. The supernatant was applied to a Streptactin gravity-flow column (1 mL bed volume) that had been equilibrated with 2x 2.5 mL buffer W as recommended by the manufacturer (IBA, Göttingen, Germany). After the sample had completely sunk into the matrix, the column was washed five times with 1 mL buffer W, 1 mL fractions were taken. The protein was eluted with 6x 0.5 mL buffer E (100 mM Tris/HCl, 1 mM EDTA, 2.5 mM desthiobiotin (pH 8.0)), 0.5 mL fractions were taken. The
column was regenerated by washing three times with 5 mL buffer R (100 mM Tris/HCl, 1 mM EDTA, 1 mM HABA (pH 8.0)) followed by removal of buffer R by washing twice with 4 mL buffer W. The presence of the respective protein in the fractions was detected by SDS-polyacrylamide gel electrophoresis analysis (12.5% Laemmli gels).

**ATP/PP<sub>i</sub>-exchange reaction**

This assay was carried out essentially as described earlier.[2] Reaction mixtures contained 200 nM enzyme, 0.5 mM amino acid, 2 mM dATP, 0.05 mM PP<sub>i</sub>, 10 mM MgCl<sub>2</sub>, 0.15 µCi [³²P]-PP<sub>i</sub> in a final volume of 100 µL buffer W, and were incubated for 10 min at 37 °C. Reactions were stopped by the addition of 500 µL ice-cold termination mix (100 mM sodium pyrophosphate, 560 mM perchloric acid, 1.2% (w/v) activated charcoal (Norit A)). The samples were vortexed and the charcoal was collected by centrifugation for 1 min at 13,000 rpm in a table-top centrifuge. The pellet was washed twice with 1 mL water and subsequently resuspended in 500 µL water. The resuspended charcoal was mixed with 3.5 mL Rotiszint Eco Plus scintillation fluid (Roth, Karlsruhe, Germany) and counted using a 1900CA Tri-Carb liquid scintillation analyzer (Packard, Dreieich, Germany).

For the determination of kinetic constants, mixtures (in triplicate) containing the enzyme and the amino acid in 50 µL buffer W were preheated at 37 °C for 1 min before the addition of the remaining components in 50 µL buffer W. The reaction was allowed to proceed for 1 min at 37 °C before the termination mix was added. The charcoal pellet was washed three times instead of twice.

To determine the functionality and specificity of the amino acyl-adenylate analogues, 5'-O-[N-(phenylalanyl)-sulfamoyl] adenosine 1 and 5'-O-[N-(leucyl)-sulfamoyl] adenosine 2 were used.
at 0.5 mM final concentration in the ATP/PP\textsubscript{i}-exchange assay. Reaction mixtures containing the inhibitor but lacking the substrate amino acid were pre-incubated at 37 °C for 1 min. Control reactions contained no amino acid or no inhibitor.

For the determination of \(K_i\)-values, the concentration of the inhibitors was varied, while the concentration of the substrate amino acid was kept constant. Measurements were made for three different concentrations of the substrate. For PheA, the concentration of L-Phe or D-Phe was 0.005, 0.01 and 0.02 mM, respectively, while the concentration of 5'-O-[N-(phenylalanyl)-sulfamoyl] adenosine 1 was varied from 0 to 0.05 µM. The concentration of L-Leu for LeuA was either 0.2, 0.5 or 2 mM while the concentration of 5'-O-[N-(leucyl)-sulfamoyl] adenosine 2 was varied from 0 to 10 nM.

For comparative studies of 5'-O-[N-(phenylalanyl)-sulfamoyl] adenosine 1 and 2'-O-[polyether-biotin]-5'-O-[N-(phenylalanyl)-sulfamoyl] adenosine 3, both compounds were used at 2, 5, 10, 50 and 100 nM final concentration in reaction mixtures (in duplicate) containing 200 nM PheA and 10 µM L-Phe in ATP/PP\textsubscript{i}-exchange assay following the procedure that was used to determine kinetic constants (see above).

**Literature**
