Exploiting Ligand Conformation in Selective Inhibition of Non-Ribosomal Peptide Synthetase Amino Acid Adenylation with Designed Macrocyclic Small Molecules

Justin S. Cisar,[†,‡] Julian A. Ferreras,[§] Rajesh K. Soni,[§] Luis E. N. Quadri,*,[†,§] and Derek S. Tan*,[†,‡]

Tri-Institutional Training Program in Chemical Biology, Tri-Institutional Research Program and Molecular Pharmacology & Chemistry Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Box 422, New York, New York 10021, and Department of Microbiology & Immunology and Molecular Biology Program, Weill Medical College of Cornell University, 1300 York Avenue, Box 62, New York, New York 10021

Received March 27, 2007; E-mail: leq2001@med.cornell.edu; tand@mskcc.org

Recent evidence suggests that natural products are much more than simply agents of "microbial warfare" and actually play critical roles in bacterial pathogenesis and communication.[1] In particular, non-ribosomal peptide (NRP) natural products have been identified as key players in bacterial iron uptake,[2] biofilm formation,[3] and commensalism.[4] Thus, small molecule inhibition of NRP biosynthesis would provide a powerful means to study the biological roles of these natural products and a potential avenue to develop novel antibiotics. Detailed mechanistic insights into NRP biosynthesis,[5] developed primarily from the perspectives of fundamental interest and engineered biosynthesis, can be leveraged to design such inhibitors. Along these lines, we recently reported a mechanism-based inhibitor of NRP siderophore biosynthesis, salicyl-AMS (5′-O-[N-salicylsulfamoyl]adenosine).[6] This compound targets salicylate adenylation enzymes by mimicking a key salicyl adenylate (salicyl-AMP) reaction intermediate through replacement of the reactive phosphate group with a stable sulfamate moiety. Related inhibitor design strategies have also been extended to other aryl acid adenylation enzymes that have no human homologs.[7] However, application of this strategy to more widely distributed *amino acid* adenylation domains is complicated by a major selectivity problem: aminoacyl-tRNA synthetases, which are used ubiquitously in *ribosomal* protein translation, catalyze nearly identical reactions (Figure 1a). Thus, simple aminoacyl-AMP analogs inhibit both classes of enzymes,[8,9] making them unsuitable for probing NRP function or as antibiotics. Herein, we report a solution to this problem using macrocyclic aminoacyl-AMP analogs (2a, 2b) that exploit pronounced structural differences between these two protein classes to inhibit an amino acid adenylation domain selectively.

To design inhibitors that selectively target NRP synthetase amino acid adenylation domains, we compared the reported ligand-bound crystal structures of a phenylalanine adenylation domain (PheA) and a phenylalanyl-tRNA synthetase (PheRS).[10] These enzymes catalyze analogous reactions involving adenylation of phenylalanine to form a phenylalanyl-AMP intermediate followed by transesterification to a peptidyl carrier protein thiol or tRNA hydroxyl, respectively (Figure 1a). However, these proteins have unrelated folds and bind their ligands in distinct conformations.[11] In the PheA structure, phenylalanine and AMP are bound in an overall "cisoid" conformation (Figure 1b). Examination of closely related aryl acid adenylation enzyme, fatty acyl-CoA ligase, and luciferase structures suggests that this cisoid ligand conformation is conserved across this family.[11,12] In contrast, in the PheRS structure, a phenylalanyl-AMP analog is bound in a "transoid" conformation (Figure 1c). Examination of all available ligand-bound aminoacyl-tRNA syn-
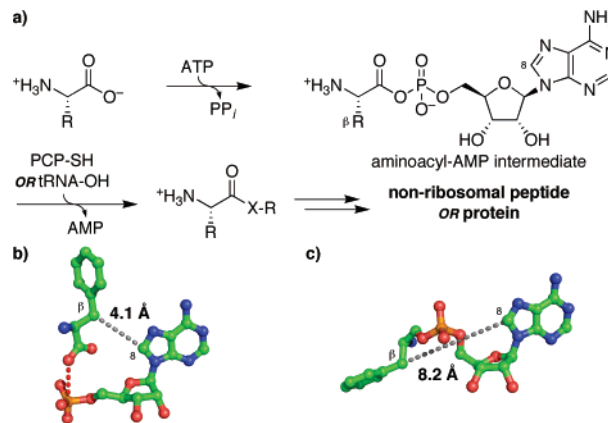
Figure 1. (a) Reactions catalyzed by amino acid adenylation domains during NRP biosynthesis (PCP-SH nucleophile) and by aminoacyl-tRNA synthetases during protein translation (tRNA-OH nucleophile). PCP = peptidyl carrier protein. (b) Cisoid conformation of phenylalanine and AMP ligands in a phenylalanine adenylation domain active site (PheA; PDB 1AMU). (c) Transoid conformation of a Phe-AMP analog in a phenylalanyl-tRNA synthetase active site (PheRS; PDB 1B7Y). Adenine C8 to phenylalanine Cβ distances are indicated (gray).

thetase structures confirmed transoid ligand conformations in all cases.[11] On the basis of this analysis, we postulated that nonhydrolyzable aminoacyl-AMP analogs in which the cisoid conformation is promoted or enforced would inhibit NRP synthetase amino acid adenylation domains selectively, without inhibiting the corresponding aminoacyl-tRNA synthetases.

Further examination of the PheA structure revealed an unobstructed 4.1 Å space between C8 of adenine and Cβ of phenylalanine (Figure 1b), suggesting that a two- or three-atom linker might be used to enforce the desired cisoid pharmacophoric conformation in a macrocyclic inhibitor. Such compounds would not be able to adopt the transoid conformation required to bind the corresponding aminoacyl-tRNA synthetases. Thus, we designed macrocycles 2a–e (Figure 2) as constrained analogs of alanyl-AMP. We envisioned that such compounds might inhibit other amino acid adenylation domains, such as the cysteine adenylation domain in the *Yersinia pestis* siderophore biosynthesis enzyme HMWP2,[13] provided that the missing β sidechain could be compensated by a reduced entropic cost of binding and/or new favorable binding interactions.[14]

After exploring several synthetic approaches, we arrived at macrocycles 2a–e using the general strategy outlined in Figure 2.[11] Briefly, 8-iodoadenosine 3 is functionalized at the adenine C8 position via cross-coupling reactions, then 5′-O-sulfamoylated. Macrolactamization of 4 and global deprotection provides macro-
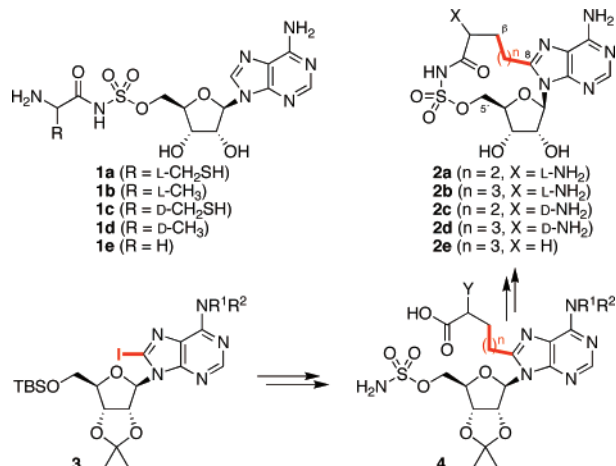
Figure 2. Structures of adenylation domain inhibitors and general synthetic approach to macrocycles.[11] (R^1, R^2 = Boc or H; Y = NHBoc or H).

Table 1. Inhibition of a Non-Ribosomal Peptide Synthetase Amino Acid Adenylation Domain and of in Vitro Translation[11]

entry	compound	K^{app} (HMWP2, μM)[a]	IC_{50} (in vitro translation, μM)[b]
1	**1a** (L-Cys-AMS)	0.24 ± 0.02[c]	15.5 ± 0.3
2	**1b** (L-Ala-AMS)	2.5 ± 0.2	0.16 ± 0.03
3	**1c** (D-Cys-AMS)	0.37 ± 0.02	n.d.
4	**1d** (D-Ala-AMS)	34 ± 3	n.d.
5	**1e** (Gly-AMS)	16 ± 5	n.d.
6	**2a** ($cyclo^{8C_3\beta}$-L-Ala-AMS)	1.7 ± 0.1[d]	>250
7	**2b** ($cyclo^{8C_3\beta}$-L-Ala-AMS)	5.4 ± 0.8[e]	>250
8	**2c** ($cyclo^{8C_3\beta}$-D-Ala-AMS)	150 ± 60	n.d.
9	**2d** ($cyclo^{8C_3\beta}$-D-Ala-AMS)	400 ± 100	n.d.
10	**2e** ($cyclo^{8C_3\beta}$-propionyl-AMS)	210 ± 30	n.d.

[a] With 30 nM HMWP2$^{1-1491}$-His$_6$, 1 mM [^{32}P]-PP$_i$, 3 mM ATP, 3 mM L-cysteine. [b] n.d. = not determined. [c] K_i^{Cys} = 0.101 ± 0.005 μM; K_i^{ATP} = 0.062 ± 0.002 μM.[11] [d] K_i^{Cys} = 0.27 ± 0.03 μM; K_i^{ATP} = 0.179 ± 0.005 μM. [e] K_i^{Cys} = 0.92 ± 0.06 μM; K_i^{ATP} = 0.57 ± 0.01 μM.

cycles **2**. Linear aminoacyl-AMS analogs **1a**–**e** were also synthesized for comparison.

With this battery of compounds in hand, we set out to test their inhibitory activities against the cysteine adenylation domain of yersiniabactin synthetase HMWP2. Gratifyingly, both macrocycles **2a** and **2b** were potent inhibitors in a cysteine adenylation assay (Table 1).[11] Notably, the two-carbon-linked macrocycle **2a** was slightly more potent than L-alanyl-AMS (**1b**) and nearly as potent as the "cognate" inhibitor L-cysteyl-AMS (**1a**). In contrast, macrocycles **2c** and **2d**, which are analogs of D-alanyl-AMS (**1d**), and desamino macrocycle **2e** were all poor inhibitors.

To test our hypothesis that the macrocyclic constraints would prevent these compounds from inhibiting aminoacyl-tRNA synthetases, we used an in vitro translation assay containing all 20 of these enzymes.[11] While both L-cysteyl-AMS (**1a**) and L-alanyl-AMS (**1b**) potently inhibited protein translation, presumably by targeting the corresponding aminoacyl-tRNA synthetases, we were pleased to find that macrocycles **2a** and **2b** showed no inhibitory activity at up to 250 μM concentration. Thus, the macrocyclic structure provides exquisite selectivity for an amino acid adenylation domain over aminoacyl-tRNA synthetases.

In summary, we have developed potent, highly selective macrocyclic inhibitors of an amino acid adenylation domain that do not inhibit aminoacyl-tRNA synthetases. We have exploited distinct ligand binding conformations to distinguish between these mechanistically related enzymes. Further studies to explore the scope of adenylation domain inhibition and the cellular activity of these

compounds and analogs thereof are ongoing. Given the high structural homology among amino acid adenylation domains,[11,12d] it will be of interest to determine whether such compounds can inhibit other domains, thereby providing a broad spectrum means to inhibit NRP biosynthetic pathways and to probe the biological and therapeutic implications thereof. Broad inhibitors might also synergistically inhibit multiple adenylation domains in an individual pathway to afford increased potency[15] and decreased susceptibility to resistance conferring mutations.[16]

Supporting Information Available: Detailed experimental procedures and analytical data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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