Distributed Drug Discovery (D3) Synthesis and Testing of Multiple Unnatural Dipeptides Identifies a Subset with Potent Antimicrobial Activity against *Pseudomonas aeruginosa* (*Pa*)

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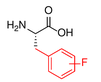
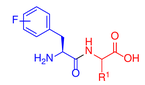
Abstract

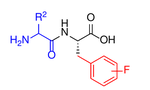
Through Distributed Drug Discovery (D3)[1] the unnatural amino acid (S)-4F-phenylalanine (**1a**: -4F) was rediscovered as a potent inhibitor of *Pseudomonas aeruginosa* (*Pa*) growth. Three close analogs (**1b**: -F; **1c**: 2-F; and **1d**: 3,4-diF) were also made and found to be active. Subsequently it was shown that unnatural dipeptides of generic structure **2** and **3** were active, presumably as prodrugs of **1**. The D3 program, which teaches solid-phase organic and peptide synthesis to students at global schools,[2,3] targeted an 80 compound subset of **2** for distributed synthesis. In this subset, the C-terminal residue is one of 20 natural amino acids and the N-terminal residue is either (*S*)-4F, 3-F, 2-F, or 3,4-diF-phenylalanine. A complementary biology laboratory was developed at IUPUI to explore the activity of these compounds against *Pa*. We report the multi-school distributed synthesis and testing of members of this targeted 80 compound subset and the resulting discovery of many unnatural dipeptides **2** active against *Pa*.



1a: 4-F; **b**: 3-F; **c**: 2-F; **d**: 3,4-diF



2: One of four N-Terminal FPhe's, R^1 = C-terminal natural or unnatural amino acid side chain



3: One of four C-Terminal FPhe's, R^2 = N-terminal natural or unnatural amino acid side chain

The D3 Vision

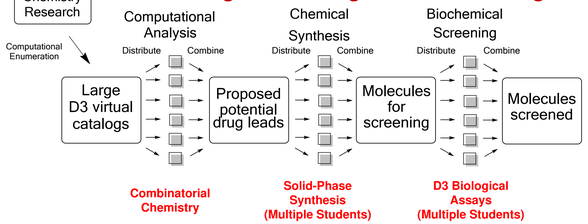
Stage One

Distributed Drug Discovery (D3) Concept[1]

J. Comb. Chem. **2009**, *11*, 3-13

D3 Directed Basic Chemistry Research

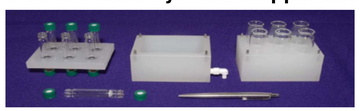
D3 Enabling Methodologies and Technologies



At each stage of the discovery process D3 breaks large challenges into smaller pieces and distributes them globally for both education and problem solving

Equipment Used: Simple Solid-Phase Synthesis Apparatus

Simple, inexpensive solid-phase synthesis Bill-Board equipment[4]

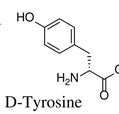


Bill-Board 6-pack | Bill-Board Drain Tray | Collection Vial Rack

D-Tyrosine analogs: Candidates for D3 *Pseudomonas aeruginosa* (*Pa*) antibiotic research?

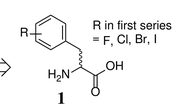
Target: Analogs of Unnatural enantiomer D-Tyrosine

D-Amino Acids Trigger Biofilm Disassembly: Kolodkin-Gal I, Romero D, Cao S, Clardy J, Kolter R, Losick R, **Science** 328, 627 (2010). (Results not reproduced: Kao et al. **Laryngoscope Investigative Otolaryngology** 2: February 2017, p. 4-9)



D-Tyrosine

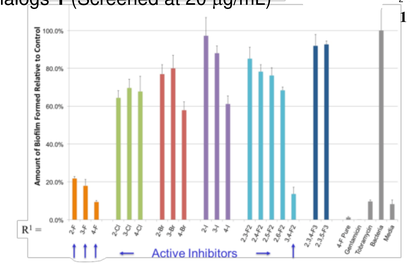
Possible drug relevance: D-tyrosine reported to modulate biofilm formation by *Pseudomonas aeruginosa*



1, Generic version of racemic tyrosine analogs accessible by O'Donnell/Scott unnatural amino acid SPS (*J Am Chem Soc* **1996,** *118*, 6070-1

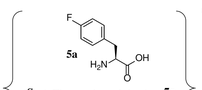
Identification of FPhe *Pa* inhibitors

Graphical Results from Student Biofilm Assay of Student-Made Racemic Tyrosine Analogs **1** (Screened at 20 μg/mL)



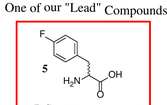
4-FPhe Hit Follow Up

Head-to-Head Comparison of 4-Fluorophenylalanine with Tobramycin

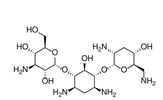


(*S*)-4-Fluorophenylalanine **5a** subsequently identified as the active enantiomer, contrary to prediction from reported D-tyrosine activity

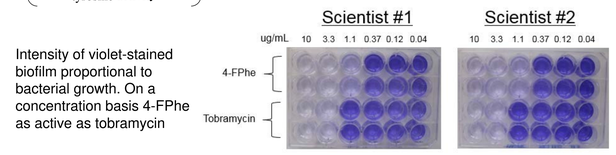
One of our "Lead" Compounds:



(*R,S*)-4-Fluorophenyl alanine ("4-FPhe")



Tobramycin
(Standard treatment for *P. aeruginosa* infections in cystic fibrosis patients)



Intensity of violet-stained biofilm proportional to bacterial growth. On a concentration basis 4-FPhe as active as tobramycin

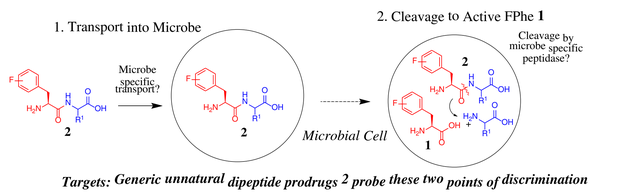
Exciting, but 4-Fluorophenylalanine Antimicrobial Activity Known in 1949!

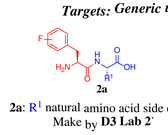
"In Vitro Effects of Metabolite Displacers on *Pseudomonas Aeruginosa*" (G.J. Martin and J.N. Moss, *Amer. J. Pharm and the Sciences Supporting Public Health*, **1949**, *121*, 169-172)

Other literature noted mammalian toxicity: How to minimize – Prodrugs?

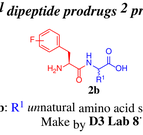
D3 Prodrugs of FPhe's

Unnatural Dipeptide Prodrugs **2** or **3**: Possible Points of Selective Toxicity for Microbes versus Mammalian Cells

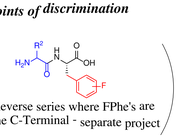




2a: R^1 natural amino acid side chain. Make by **D3 Lab 2**[*]



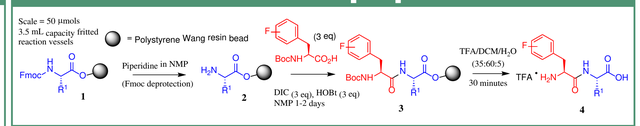
2b: R^1 unnatural amino acid side chain. Make by **D3 Lab 8**[*]



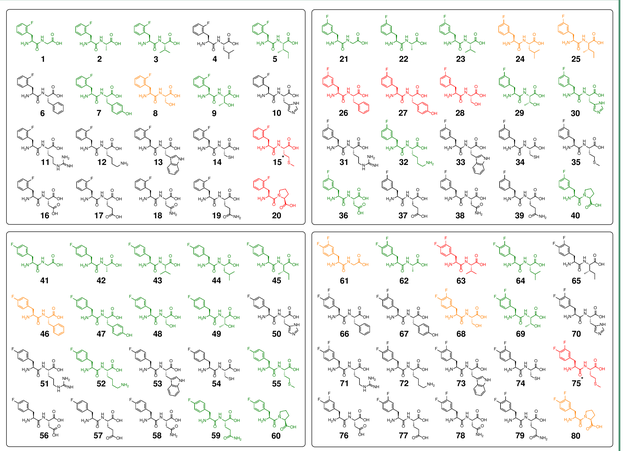
3: Reverse series where FPhe's are at the C-Terminal - separate project

Goal: Distributed Synthesis/Testing of 80 Compounds 2a (four FPhe's x 20 AAs)

D3 Unnatural Dipeptide Lab 2



80 Compound 2a Target Quadrants with Test Results



At 10 μg/mL, % biofilm formation relative to bacteria control: Active = <10%; marginal = 11-30%; inactive = >31% (black = not yet made or made but not tested)

[*] Samples for **15** and **75** very impure (30-40%) by LC/MS

Synthesis/Test Results

- Values are % bacteria biofilm growth
- Replicates highlighted by color
- Lot # "ave…." is the average of 2-10 lots from C344 2019 lab
- Most unpurified lots > 80% pure by LC/MS
- All Colorado College ("CC") lots purified
- Early testing at 10 μg/mL, later down to 0.5 μg/mL

Made but not yet tested:

Santa Clara University: 21,24,28,31,32,35,37,40,41 44,48,51,52,55,57,60,61,64 68,71,72,75,77,80

Goshen (UIndy replicates): 21,22,23,25,32,39,40,41,42 43,45,52,59,60

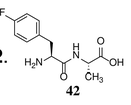
Synthesis in progress:

U of Puerto Rico: 22,24,26, 42,44,46

UNAM (Mexico City): 22, 26,27,28,40,42,46,47,48,60

Best So Far

From set made and tested most active compound is **42**. Estimated IC_{50} = 0.5 μg/mL



42

Other D3 Goals

- Emphasize Replication

Project Cmpd#	School	Lot #	TFA salt?	10 ug/mL	5 ug/mL	1 ug/mL	0.5 ug/mL
42	IUPUI	Ave C344 SP19	Yes	0%			
42	IUPUI	F18-T01-B1	Yes	2%		37%	
42	CC	CH-6-A1	No		2%	9%	42%
42	CC	BW-6-A1	No		2%	4%	22%
42	Uindy	UI-S19-T1-A1	Yes	1%		3%	
42	Uindy	UI-S19-T2-A1	Yes	4%		46%	
42	Uindy	UI-S19-T3-A1	Yes	5%		53%	
42	Uindy	UI-S19-T4-A1	Yes	4%		28%	
42	Uindy	UI-S19-T5-A1	Yes	-1%		14%	
42	Uindy	UI-S19-T6-A1	Yes	2%		80%	

e.g. Compound **42** (4-FPhe-Ala) made by multiple researchers and each lot tested by multiple biologists

- Teach Chemistry

Students learn solid-phase organic or peptide synthesis through making unnatural dipeptides **2a** in regular organic synthesis teaching labs or while doing independent research

- Teach Biology

Students in regular microbiology lab learn about bacterial growth while testing new compounds as potential antibiotics. Independent student researchers study compound biological activity in more detail

- Teach Drug Discovery

Students learn the collaborative interdisciplinary research process critical to drug discovery. They participate in drug discovery for under-researched diseases, in this project the search for drugs to treat *P. aeruginosa* infections

- Collaborate Globally

D3 seeks to harness the power of globally distributed research to expand resources while uniting scientists around a common humanitarian challenge. Outside the continental United States this project added Puerto Rico and Mexico to the D3 collaborative network.

Summary and Conclusions

Summary and Conclusions:

- D3 Synthesis and testing of subset of 80 target prodrugs identifies many potent *Pa* inhibitors
- Activity likely a function of documented transport[5] of dipeptides into *Pa* with subsequent enzymatic cleavage
- Most active quadrant has N-terminal 4-FPhe, consistent with activity of 4-FPhe itself
- Data (not shown) on unnatural FPhe N-terminal dipeptides with *unnatural C-terminal AA's* shows critical nature of this residue - many close analogs are inactive (e.g. 4-FPhe-D-Ala, 4-FPhe-L-Pip, 4-FPhe-L-Phg are inactive at 10 μg/mL)

Important Qualifications: There is no indication these molecules directly inhibit biofilm growth – instead they inhibit biofilms by inhibiting bacterial growth. Also, activity of FPhe's is only seen in the absence of Phe in the media (Phe antagonizes activity - literature indicates FPhe's are antimetabolites acting through Phe pathways)

Challenges: 1) Coordinating multiple sites 2) Collecting and curating compounds – physically and electronically 3) Synthesizing compounds with problematic amino acids

References and Acknowledgements

References:

1. *J. Comb. Chem.* **2009**, *11*, 1-13; 14–33
2. *J. Chem. Educ.* **2015**, *92*, 819–826
3. *J. Chem. Educ.* **2019**, *96*, 1731-1737
4. Bill-Board from Chemglass Life Sciences CG-1869
5. PLoS One. 2014; 9(10): e111311. doi: 10.1371

Funding:
NSF (DUE/1140602); NIH (GM-28193); IUPUI and D3 Fund; ACS Global Innovation Grant

Analytical Support: Lilly Research Laboratories

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Student Researchers:
Multiple chemistry and biology students too numerous (>2000) to list individually