Development of Siderophore-Antibiotic Conjugates with Protease-Cleavable Linker

2023.09.30 Literature Seminar

B5 Mizuki Sawada

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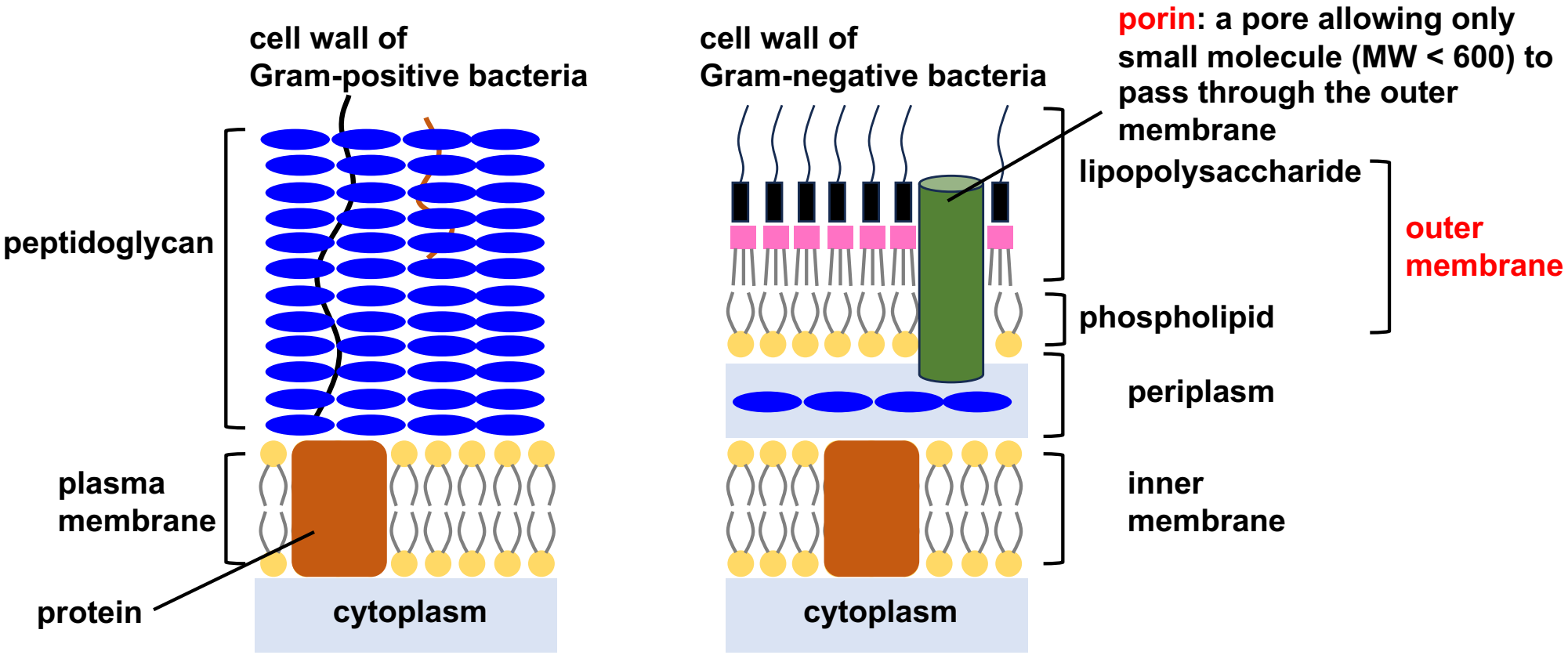
Gram-Negative Bacteria



Infection caused by multi-drug resistant Gram-negative bacteria is problematic worldwide. Development of new antibiotics is necessary but difficult because their outer membrane prevents antibiotics from reaching their target.

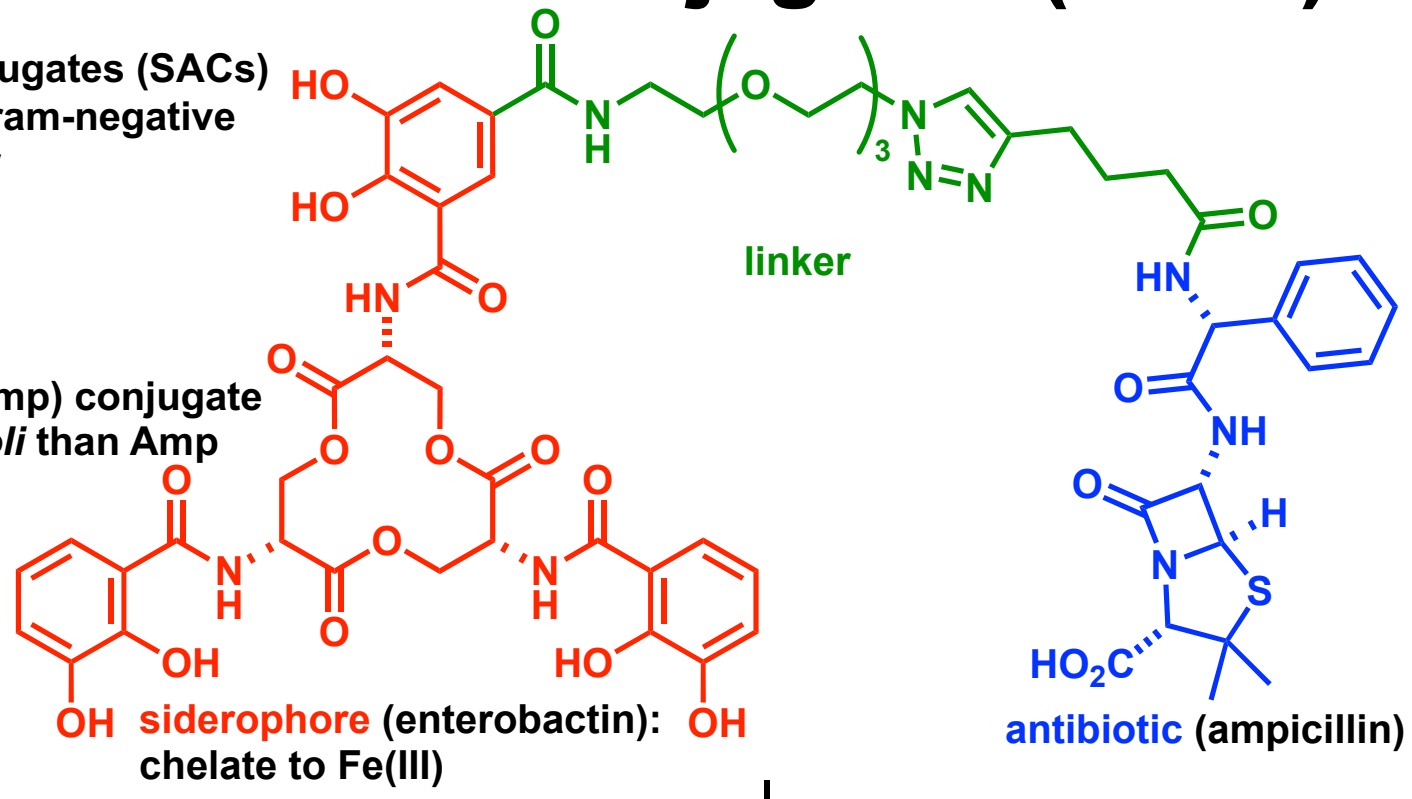
One way to convey drugs into Gram-negative spp. is to conjugate antibiotics with molecules actively taken by such bacteria.

1) Pendleton, J. N.; Gorman, S. P. and Gilmore, B. F. *Expert Rev. Anti-Infect. Ther.* **2013**, *11*, 297.
2) Breijyeh, Z.; Jubeh, B.; Karaman, R. *Molecules*. **2020**, *25*, 1340.

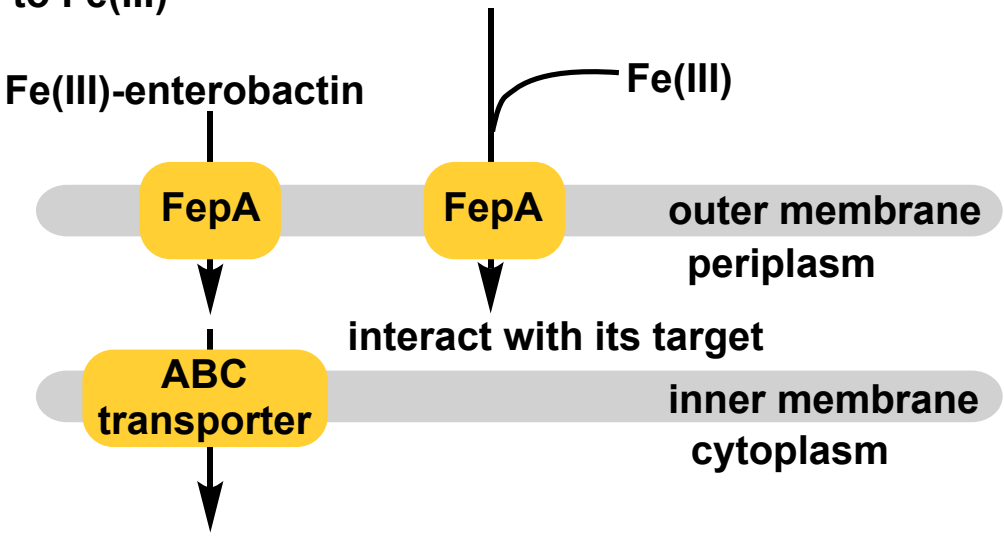
Siderophore-Antibiotic Conjugates (SACs)

Siderophore-antibiotic conjugates (SACs) can deliver antibiotics to Gram-negative bacteria through their outer membrane barrier.

- an example of SACs [1]:
enterobactin-ampicillin (Amp) conjugate
1000-fold effective to *E. coli* than Amp



- Siderophores:
high affinity to Fe(III)
produced by bacteria to get Fe(III)
- Fe(III)-siderophore complexes:
actively taken up by bacteria
- Fe(III)-SAC complexes are transported via same machinery.



1) Zheng, T. and Nolan, E. M. *J. Am. Chem. Soc.* **2014**, *136*, 9677.

Classification of SACs

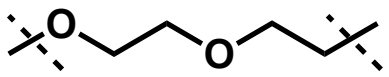
SACs

Classification by siderophore: catecholate, hydroxamate, mixed ligand, etc.

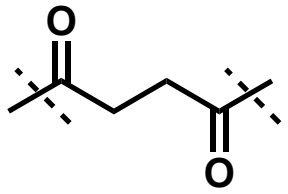
Classification by target of antibiotic: periplasm-targeting or cytoplasm-targeting
- **periplasm: β-lactam type, lipopeptide type, etc.**
- **cytoplasm: quinolone type, oxazolidinone type, etc.**

Classification by linker: non-cleavable or cleavable

non-cleavable linker

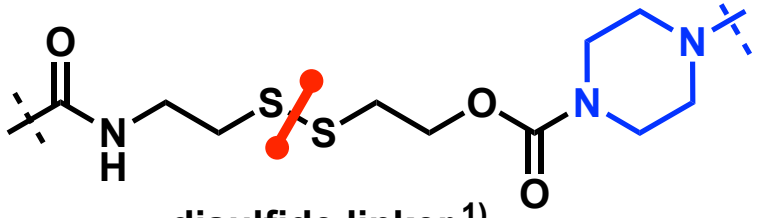


polyethylene glycol (PEG) linker

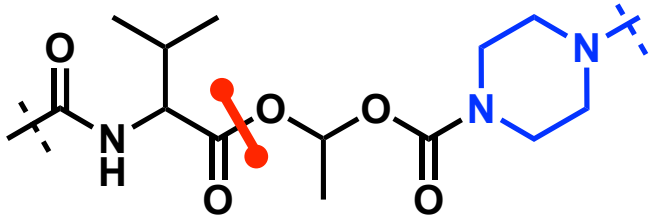


succinate linker

cleavable linker



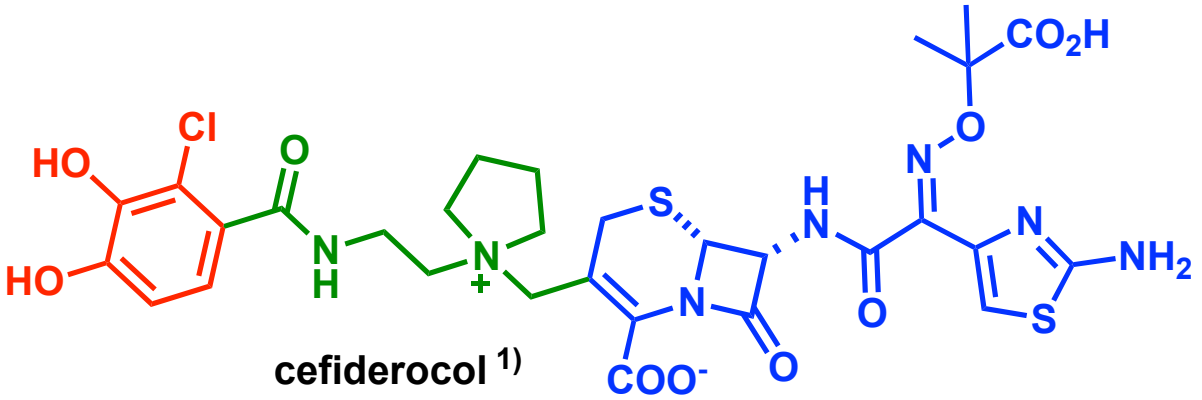
disulfide linker [1]
cleaved by reducing agents such as glutathione



ester linker: cleaved by esterase [2]

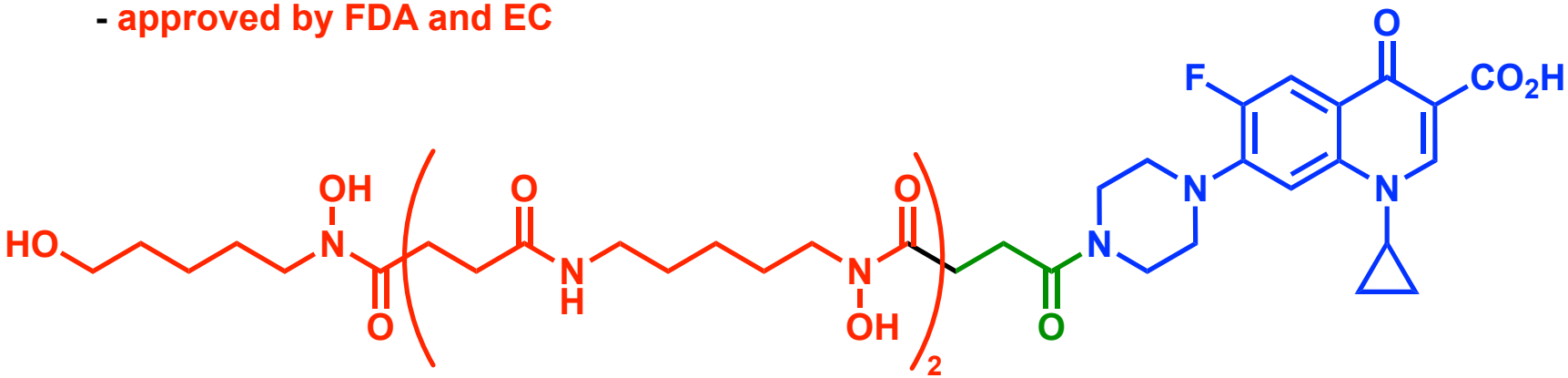
1) Neumann, W. and Nolan, E. M. *J. Biol. Inorg. Chem*. **2018**, *23*, 1025.
2) Zheng, T. and Nolan, E. M. *Bioorg. Med. Chem Lett.* **2015**, *25*, 4987.

SACs with Non-Cleavable Linkers



cefiderocol [1)]

- conjugate with periplasmic-targeting cephem antibiotic
- active against Gram-negative bacteria including carbapenem resistant strains.
- approved by FDA and EC

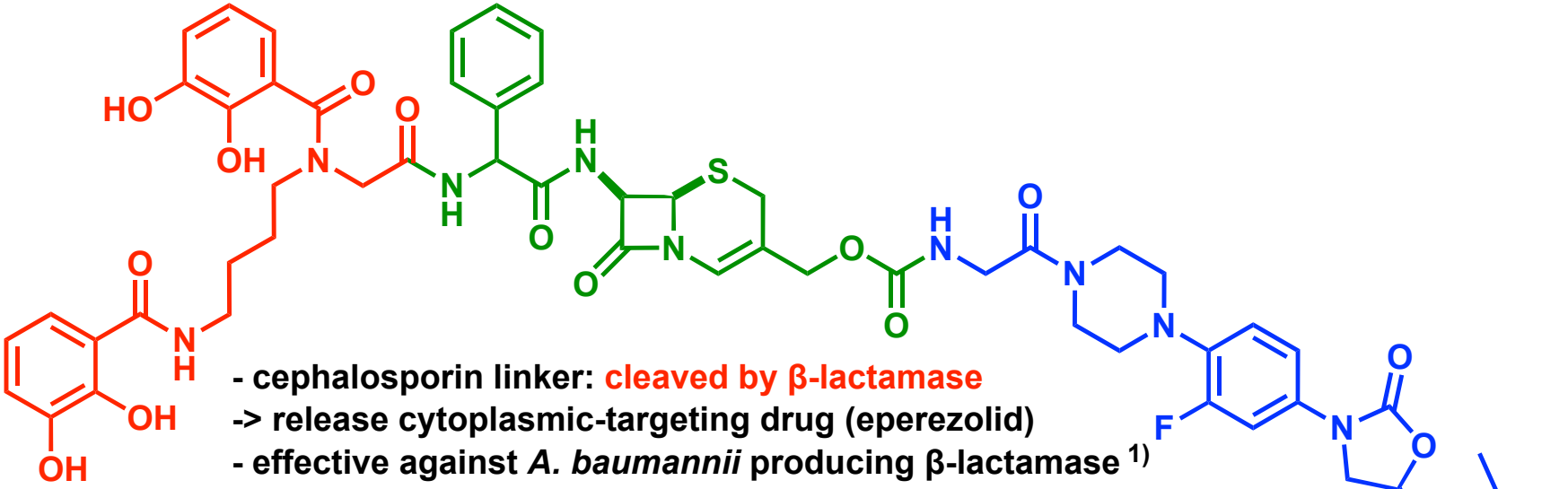


- conjugate with cytoplasmic-targeting ciprofloxacin [2)]
- inactive against Gram-negative bacteria

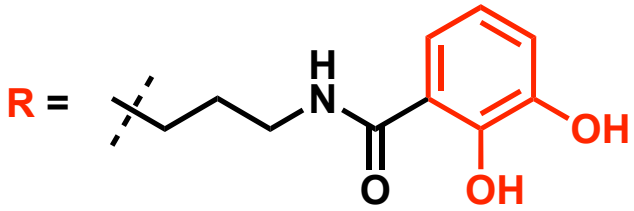
Non-cleavable SACs is efficient for periplasmic-targeting drugs.
However, the SACs conjugated cytoplasmic-targeting drugs via non-cleavable linkers are less active than their parent drugs in many cases.
The conjugates may not pass through inner membrane or interfere with binding with targets.

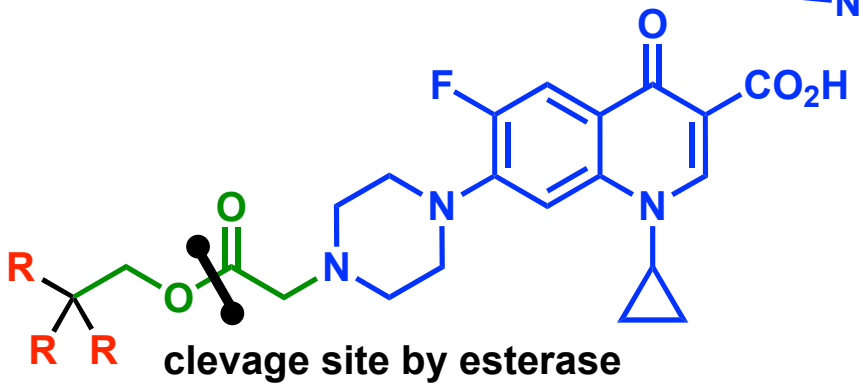
1) Aoki, T.; Yamawaki, K.; Sato, T.; Nishitani, Y. and Yamano, Y. *Medchem News.* **2021**, *31*, 75.
2) Wencewicz, T. A.; Long. T. E.; Möllmann, U. and Miller, M. J. *Bioconjugate Chem.* **2013**, *24*, 473.

SACs with Cleavable Linkers



- cephalosporin linker: cleaved by β-lactamase
-> release cytoplasmic-targeting drug (eperezolid)
- effective against *A. baumannii* producing β-lactamase [1)]





clevage site by esterase

- MIC against *P. aeruginosa*: 8 μg/mL, less effective than ciprofloxacin (0.25 μg/mL) probably due to the insufficient hydrolysis [2)]

More studies about cleavable linkers for SACs are needed.

1) Liu, R.; Miller, P. A.; Vakulenko, S. B.; Stewart, N. K.; Boggess, W. C. and Miller, M. J. *J. Med. Chem*. **2018**, *61*, 3845.
2) Fardeau, S.; Dassonville-Klimpt, A.; Audic, N; Sasaki, A.; Pillon, M.; Baudrin, E.; Mullié, C. and Sonnet, P. *Bioorg. Med. Chem.* **2014**, *22*, 4049.

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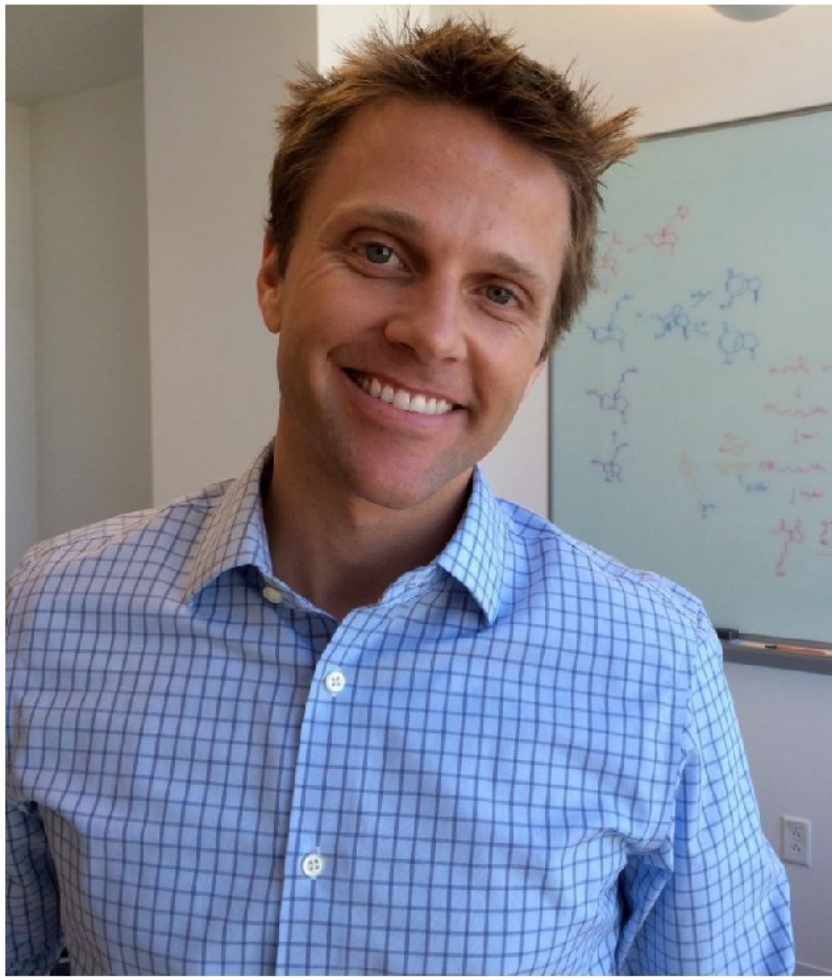
Associate Prof. Ian B. Seiple



2006 B.Sc. @ University of California, Berkeley
(Prof. Dirk Trauner)
2011 Ph.D. @ The Scripps Research Institute
(Prof. Phil Baran)
2011- Postdoctoral fellow @ Harvard University
(Prof. Andrew Myers)

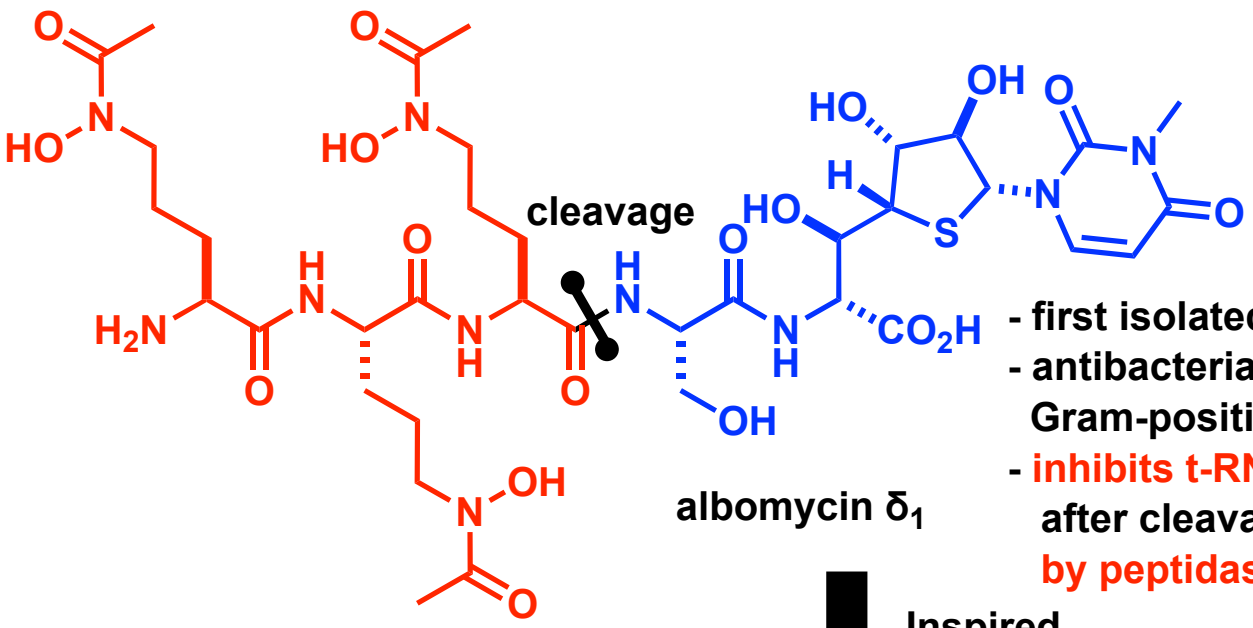
Associate Prof. @ UCSF

Research Area:
chemical biology and medicinal chemistry

https://seiplegroup.ucsf.edu/index.php/people/ian-b-seiple/
https://pharmacy.ucsf.edu/ian-seiple

Protease-Cleavable SACs

natural SAC: albomycins

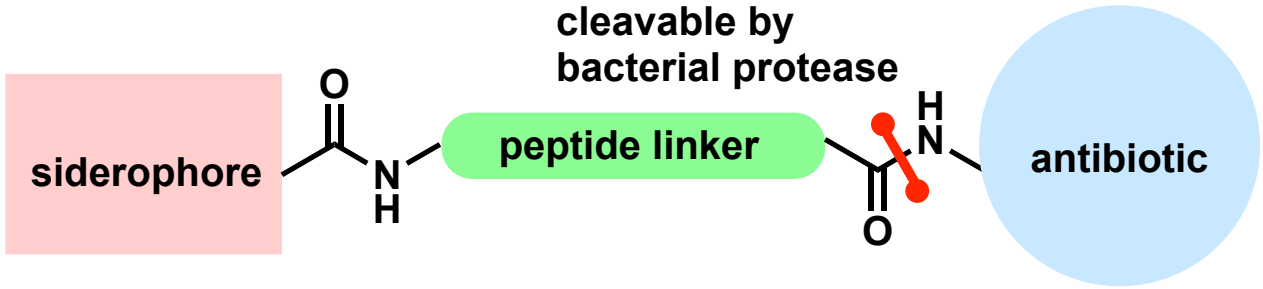


albomycin δ_1

- **first isolated from *Streptomyces griseus* [1)]**
- **antibacterial activity against both Gram-positive and -negative species**
- **inhibits t-RNA synthetase after cleavage of siderophore moiety by peptidase N [2)]**

Inspired

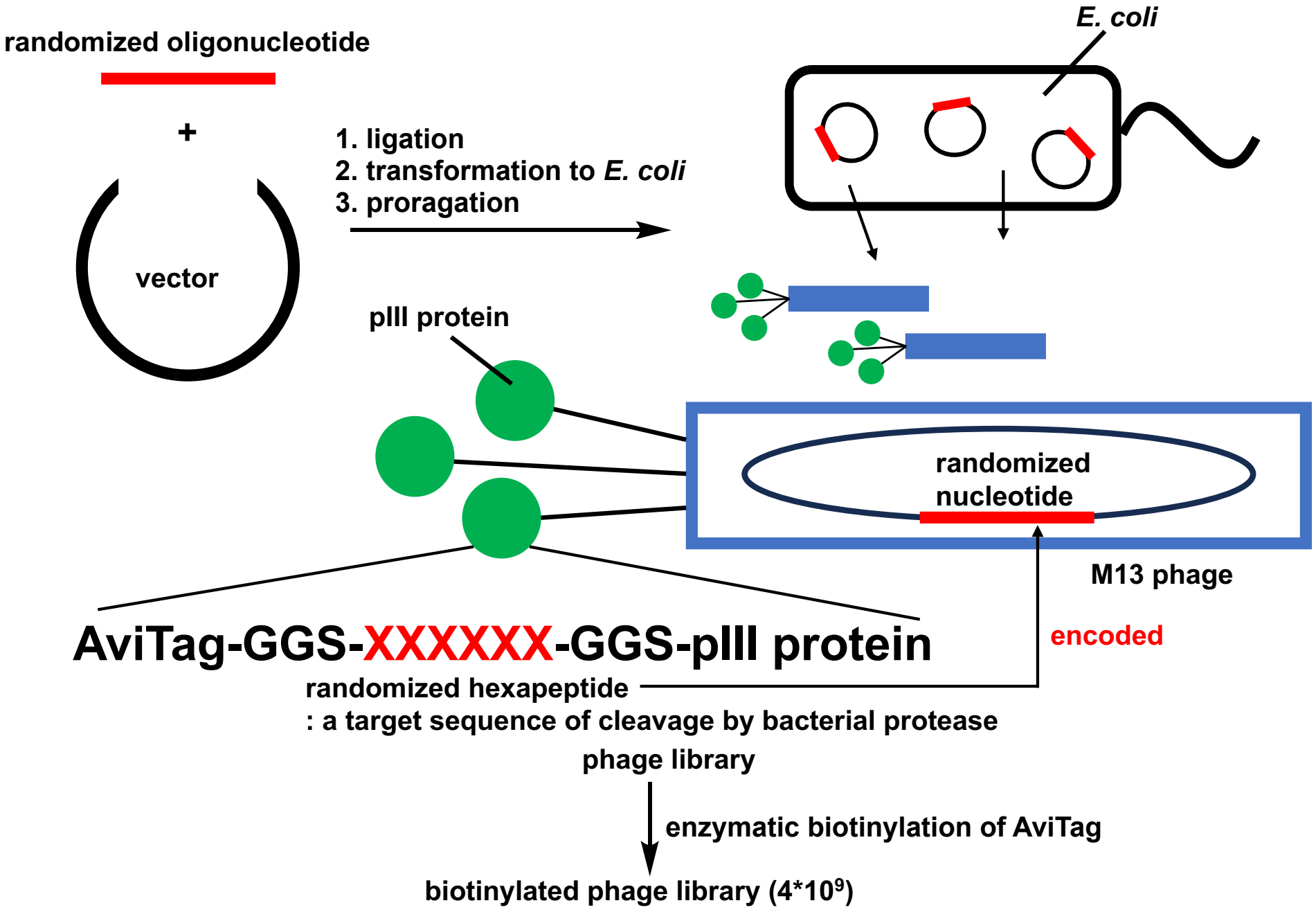
cleavable by bacterial protease



Phage display was used for screening of candidates of linker sequences, which is a method to detect the interaction with proteins and other molecules using phage library.

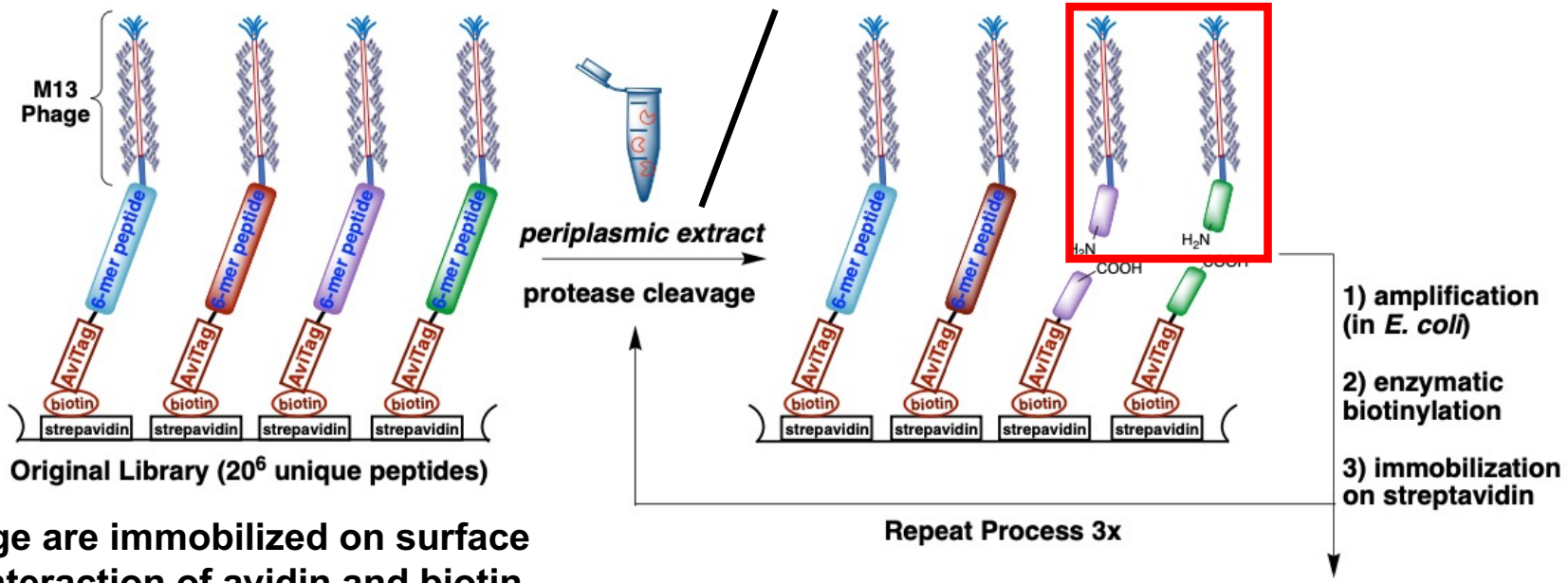
1) Reynolds, D. M.; Schatz, A. and Waksman, S. A. *Proc. Soc. Exptl. Biol. Med.* **1947**, *64*, 50.
2) Braun, V.; Pramanik, A.; Gwinner, T. Köberle, M. and Bohn, E. *Biometals,* **2009**, *22*, 3.

Construction of Phage Library

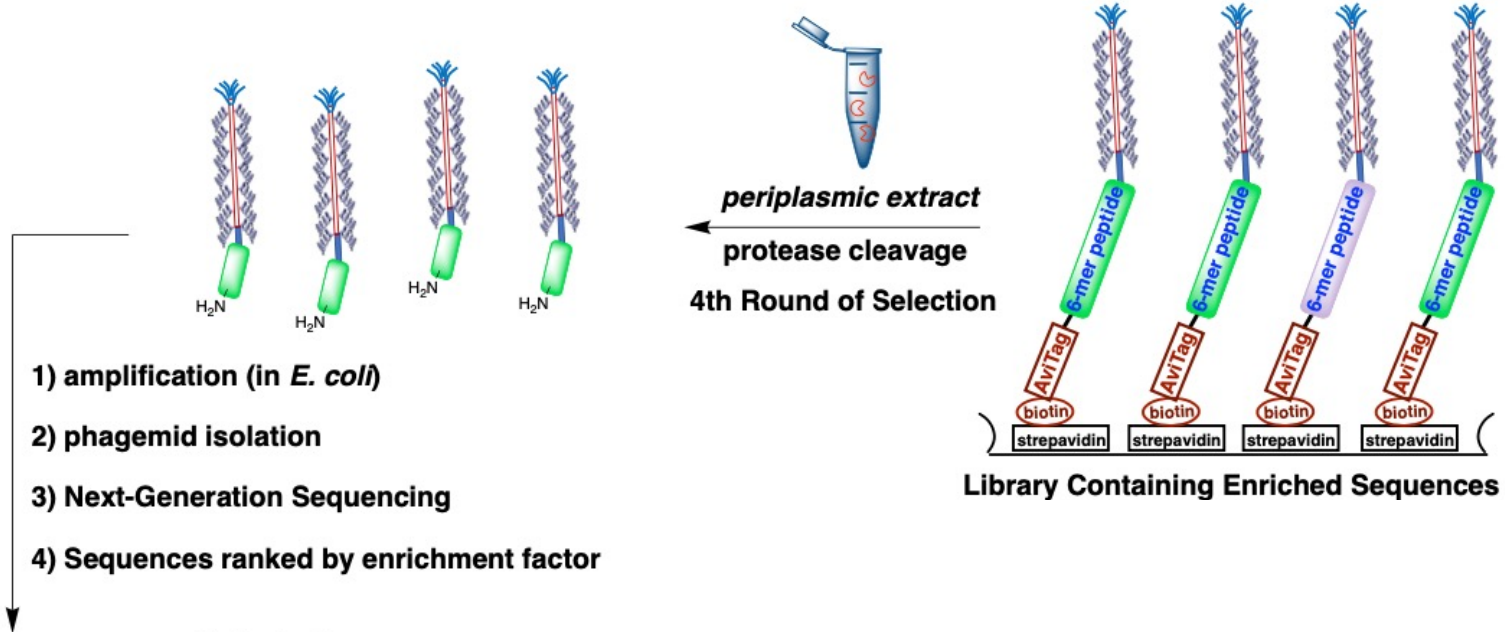


Selection of Candidate Sequences

Periplasmic extract, a mixture of protease, was used to suppress the resistance arising from mutant of a single protein



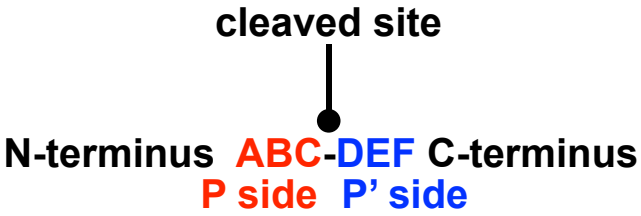
Phage are immobilized on surface by interaction of avidin and biotin.

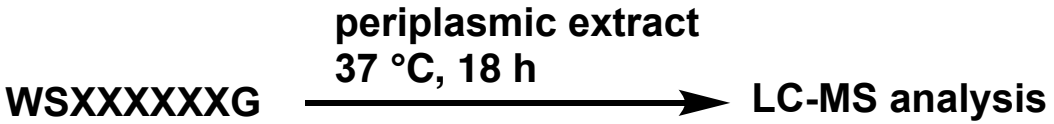


Evaluation of Selected Sequences

Selected six sequences based on enrichment factor (= output reads / initial reads):
KNQSLG, GSDSSV, NHADVH, KSEMLS, WCKWAS, PKYMRF

The cleavage site and extent of sequence was evaluated using synthesized peptides WSXXXXXXG.





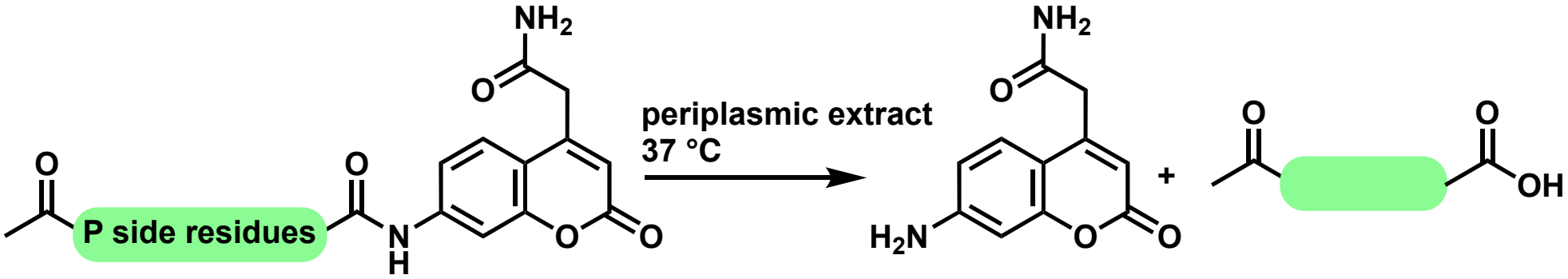
peptide	P side	P' side	uncleaved parent peptide
WSKNQSLGG	W (major)	SKNQSLGG (major)	50%
WSGSDSSVG	W (major)	SGSDSSVG (major)	50%
WSNHADVHG	WSNHA (major)	SNHADVHG (major)	30%
WSKSEMLSG	not determined	MLSG (major) SKSEMLSG (minor)	20%
WSWCKWASG	WSWC (major)	KWASG (minor)	3%
WSPKYMRFG	WSPKYM (minor)	RFG (major) YMRFG and MRFG (minor)	30%

WSWC-KWASG and WSPKYM-RFG were selected.

Validation of Linkers with Turn-On Fluorophore

Selected sequences: WSWC-KWASG and WSPKYM-RFG

Question: Can peptides with residues in P side be cleaved by protease?

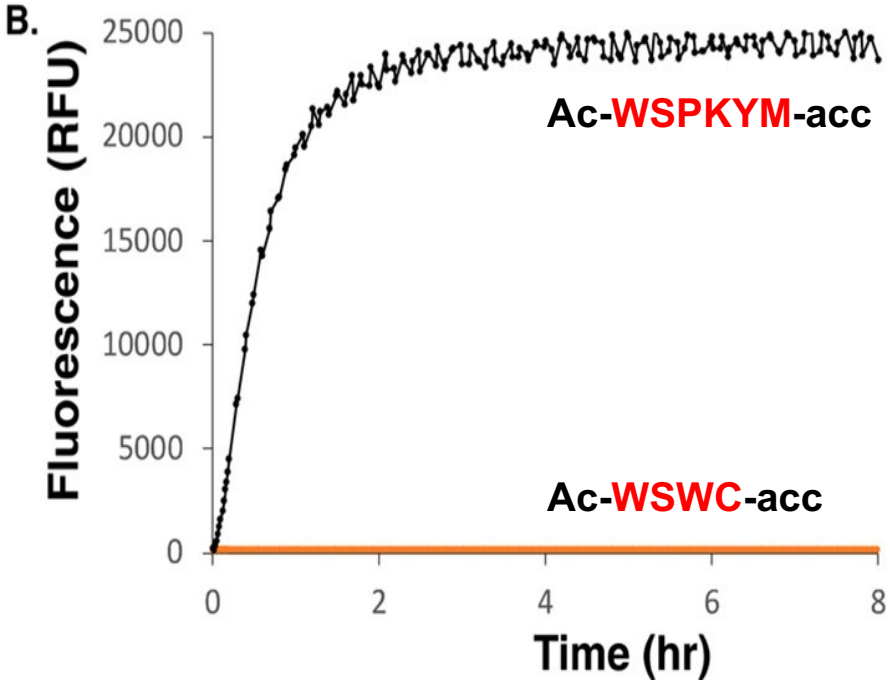


Ac-peptide-acc
(prepared by solid-phase synthesis)

non-fluorophore

7-amino-4-carbamoylmethylcoumarin (acc)

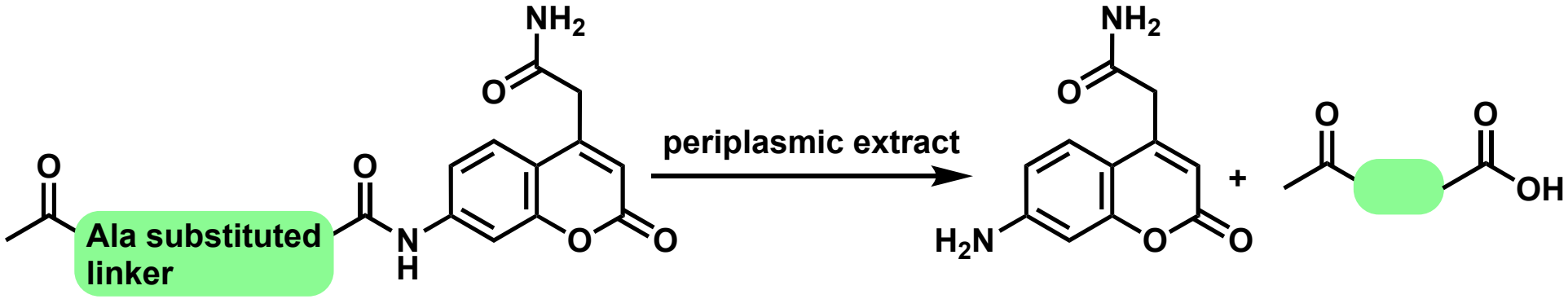
fluorophore



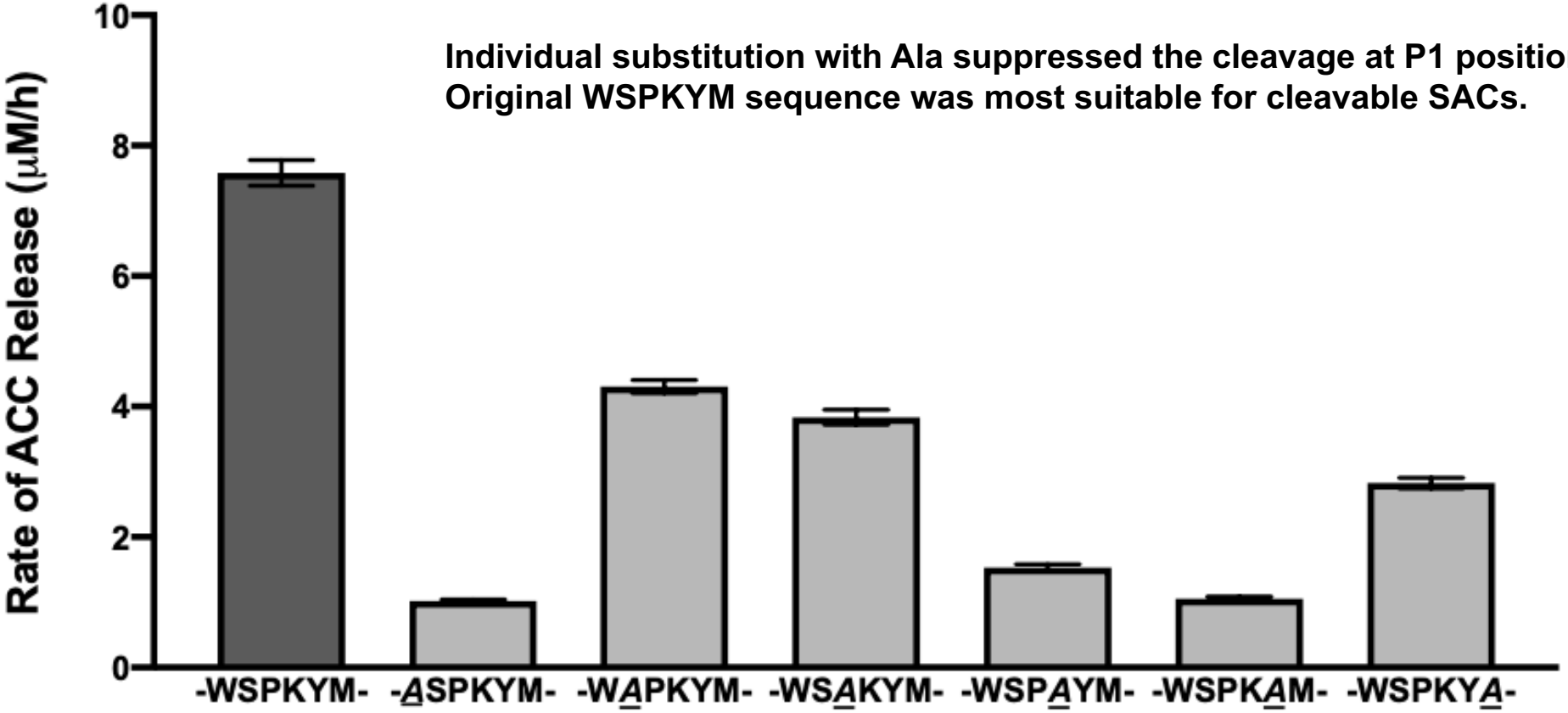
- The increase of fluorescence indicates that the desired cleavage occurred between P1 residue and acc.

WSPKYM was determined to be more suitable than WSWC for the development of cleavable SACs.

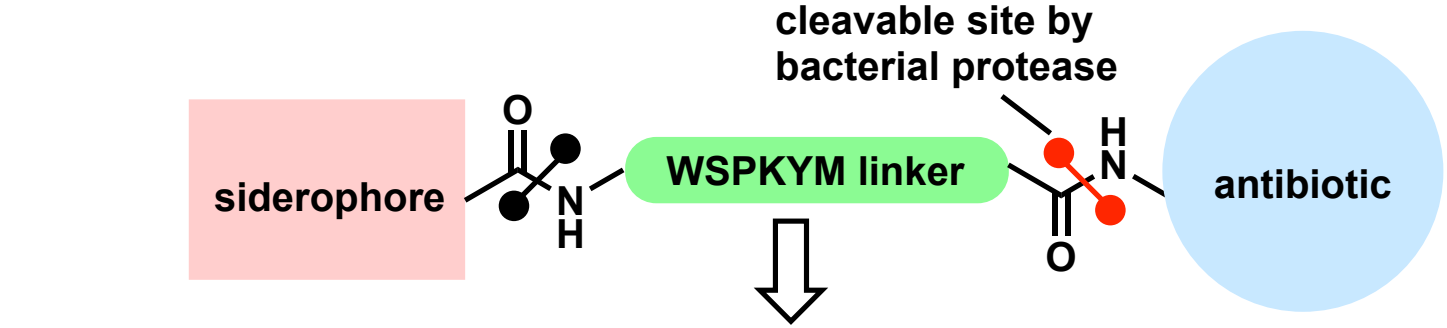
Evaluation of Sequence Dependence for Effective Cleavage

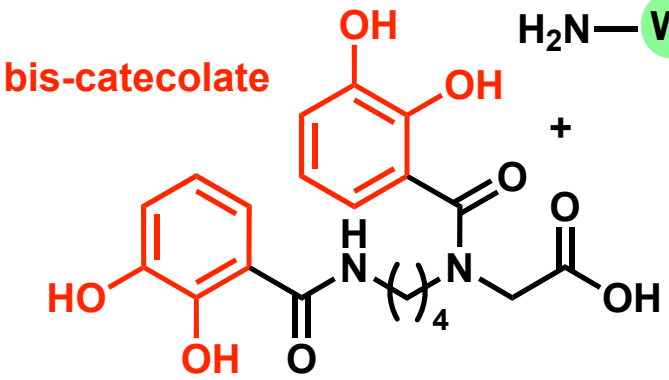


Individual substitution with Ala suppressed the cleavage at P1 position.
Original WSPKYM sequence was most suitable for cleavable SACs.



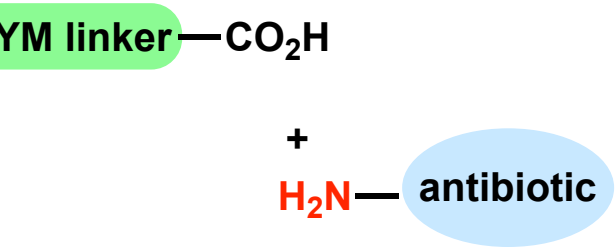
Design and Synthetic Strategy of SACs



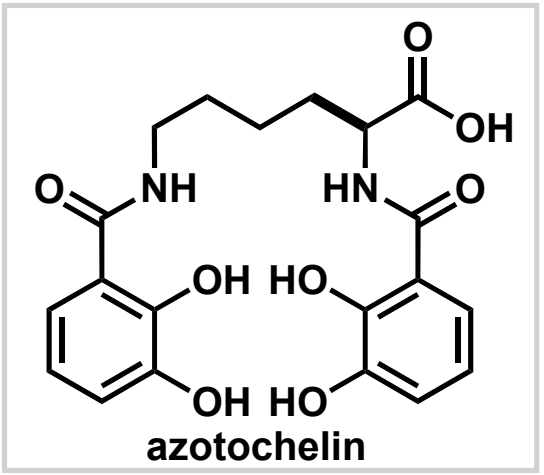


azotochelin-like siderophore

able to large cargo (e.g. daptomycin) into
A. baumannii, *E. coli*, *P. aeruginosa* [1)]



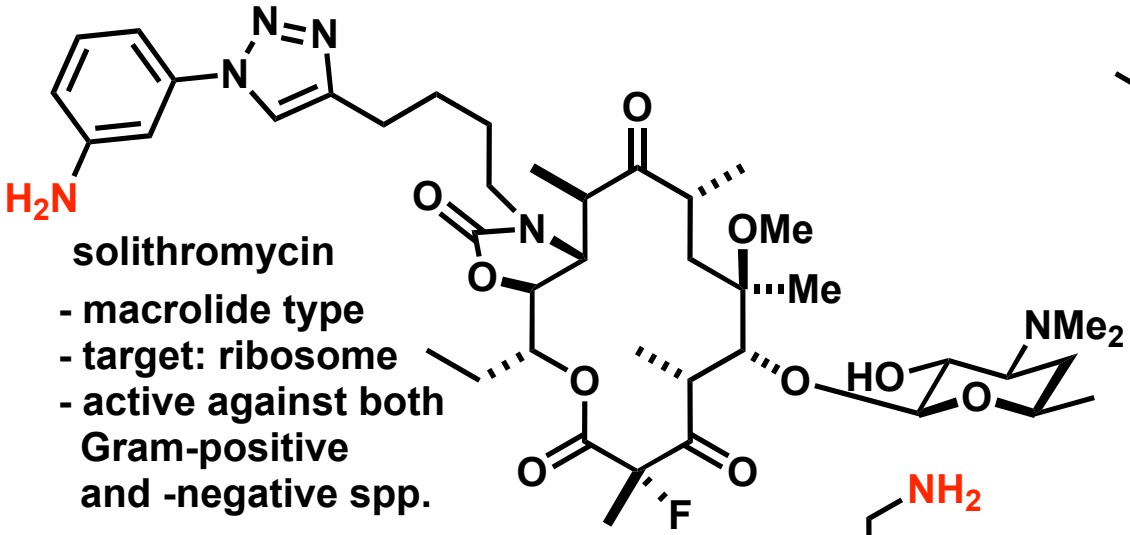
containing NH_2 group
-> Unmasked antibiotics can be released
by cleavage of linker by bacterial protease.



azotochelin

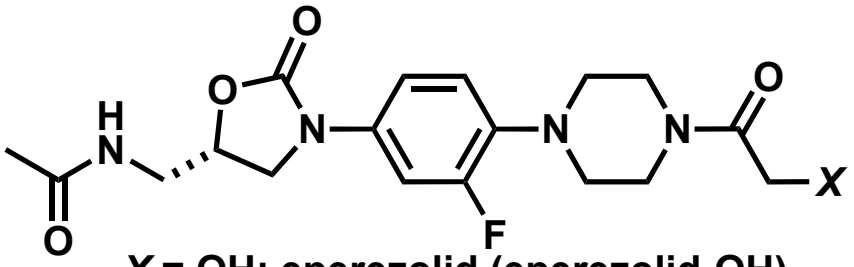
1) Liu, R.; Miller, P. A.; Vakulenko, S. B.; Stewart, N. K.; Boggess, W. C. and Miller, M. J. *J. Med. Chem.* **2018**, *61,* 3845.

Antibiotics



solithromycin

- macrolide type
- target: ribosome
- active against both Gram-positive and -negative spp.

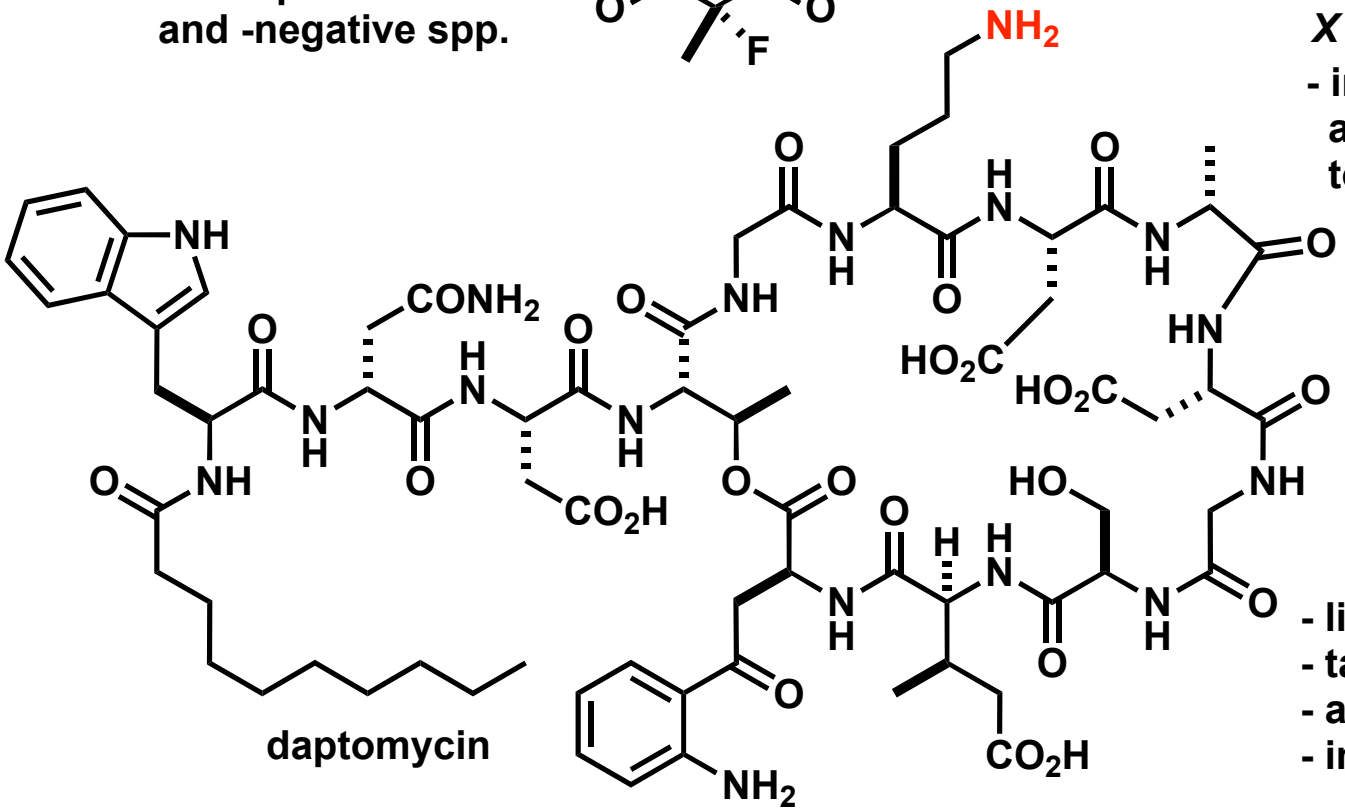


X = OH: eperezolid (eperezolid-OH)

- oxazolidinone type
- target: ribosome
- active against Gram-positive spp.
- inactive against Gram-negative spp. due to efflux pump

X = NH_2: eperezolid-NH_2

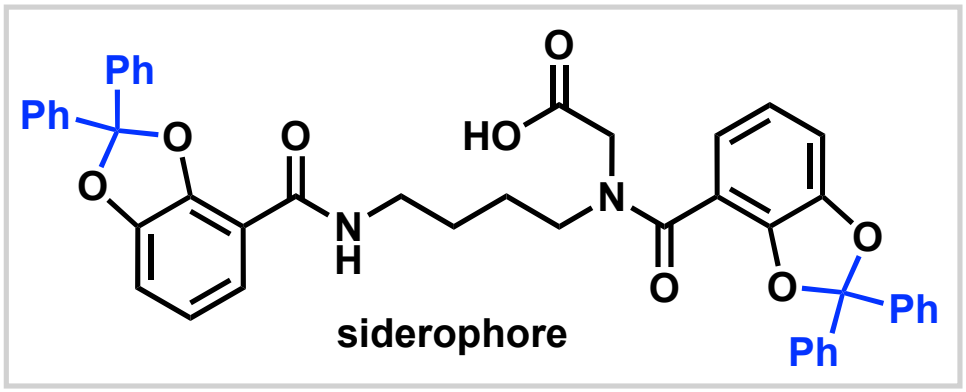
- inactive against both Gram-positive and -negative spp. due to the inability to diffuse through membranes

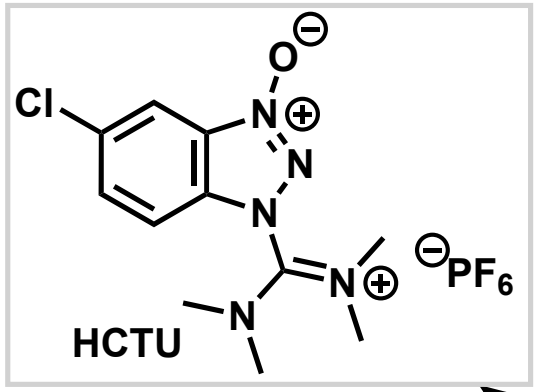


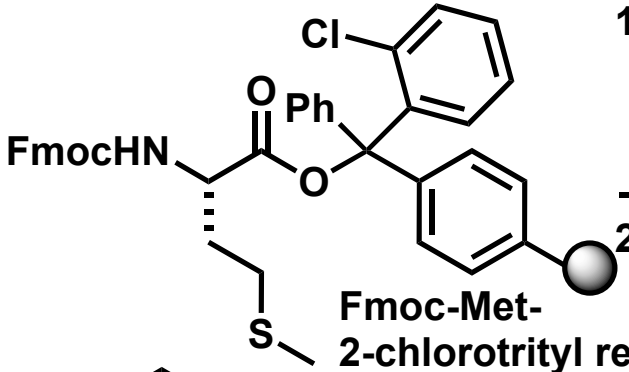
daptomycin

- lipopeptide type
- target: cytoplasmic membrane
- active against Gram-positive spp.
- inactive against Gram-negative spp.

Synthesis of SACs (1)

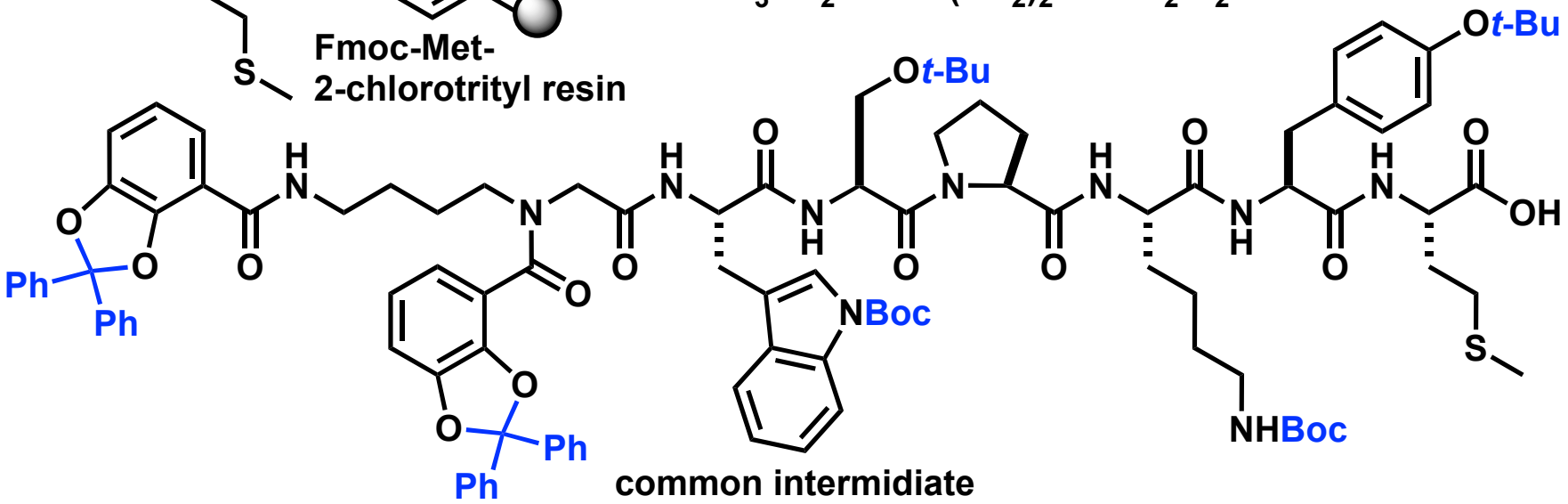






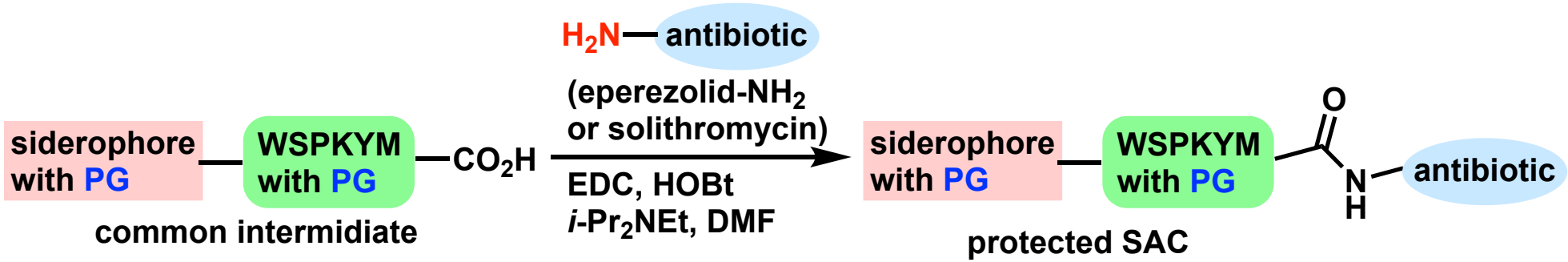
1. solid-phase synthesis (6 cycles)
   a) 4-metylpiperidine/DMF (1/4)
   b) amino acid or siderophore
      HCTU, *i*-Pr_2NEt, DMF

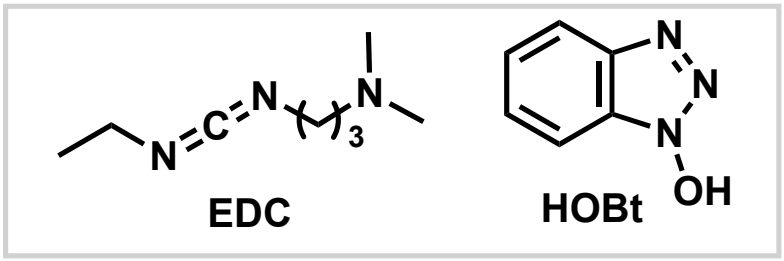
2. $AcOH/CF_3CH_2OH/HS(CH_2)_2SH/CH_2Cl_2$



= siderophore with PG — WSPKYM with PG — CO_2H

Synthesis of SACs (2)

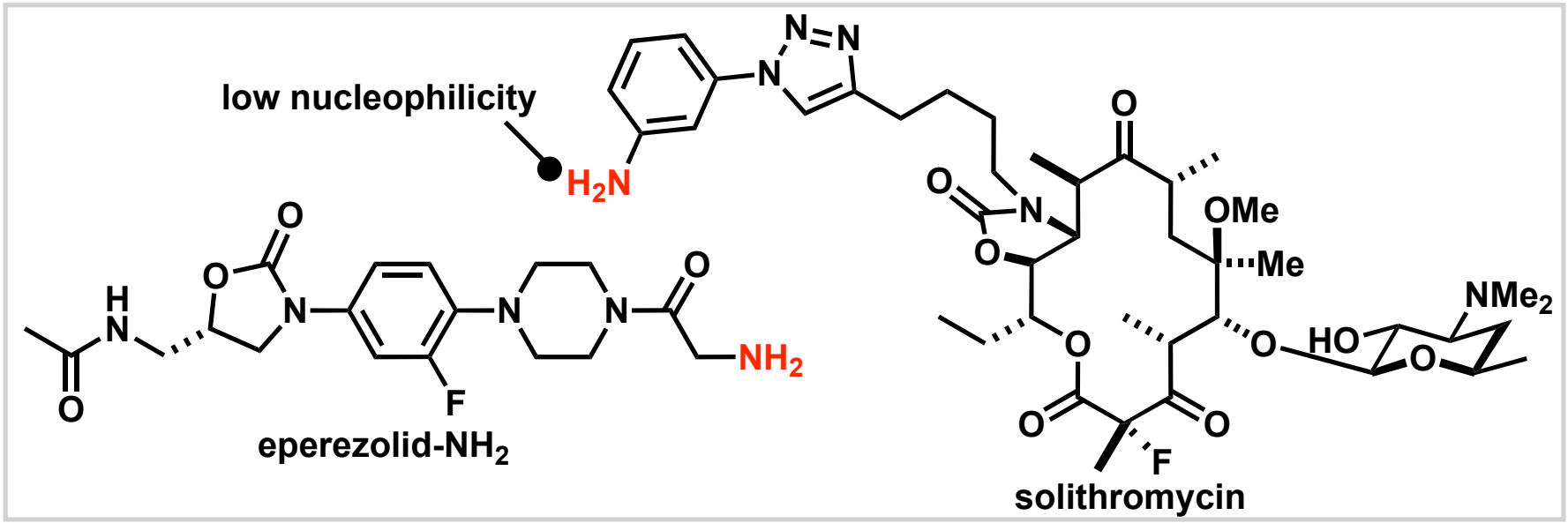




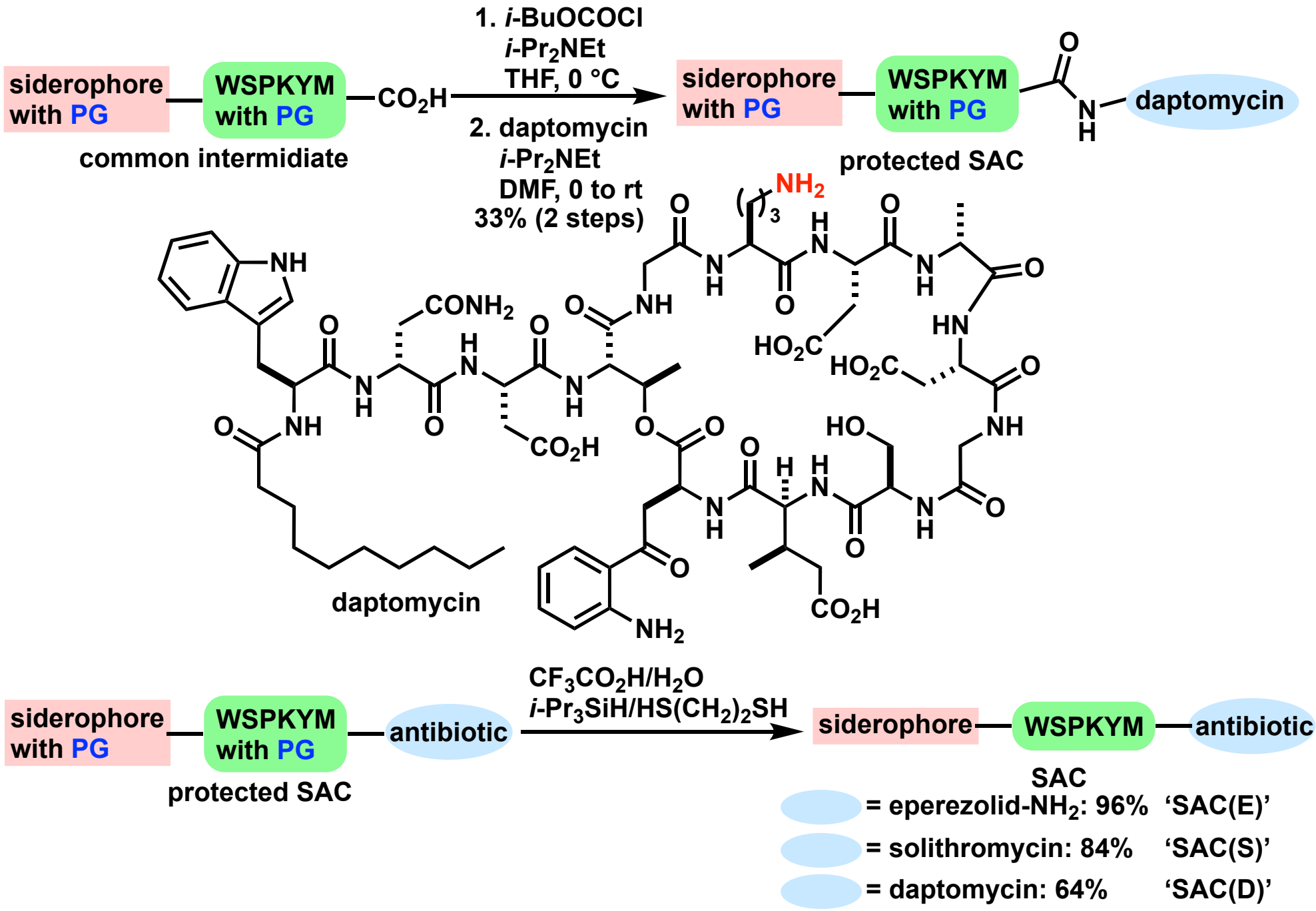
= eperezolid-NH_2, protected SAC: 55%

= solithromycin

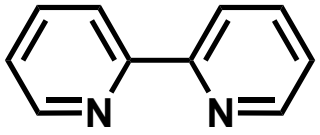
protected SAC: 16%
a diastereomer of protected SAC: 12%
(probably an epimeric compound at C_α position of C-terminus Met produced via azlactone)
SM rcv.: 45%



Synthesis of SACs (3)

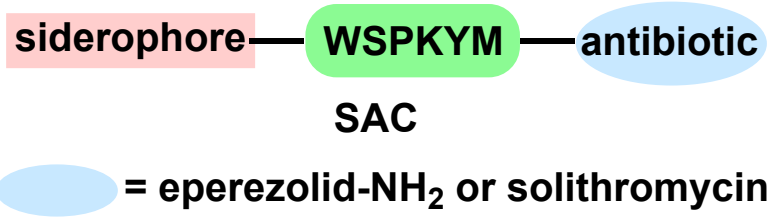


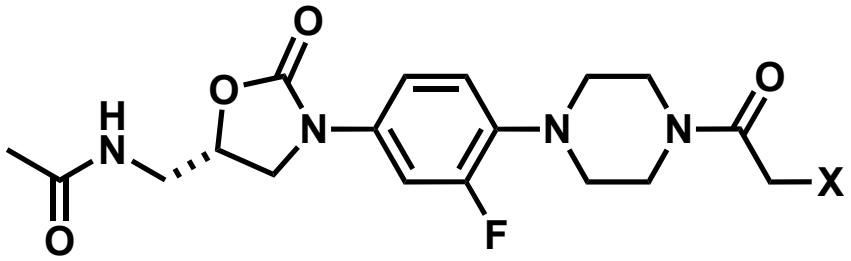
Iron-Dependent Antibacterial Activity



2, 2'-dipyridyl (DP)

- Fe(II) chelator
-> added to medium to generate iron-deficient conditions and promote expression of siderophore transport machinery





eperezolid-X (X = NH_2 or OH)

Antibacterial activity against *E. coli* Δ*bamB*Δ*tolC* [a] (MIC, μM)

concentration of DP [μM]	0	129	200
SAC(E)	19	5	1
eperezolid-OH	5	5	5
eperezolid-NH_2	>171	>171	>171

[a] lacking the efflux pump

MIC color scale

1	3	5	11	19	23	48	>48

MIC = minimum inhibitory concentration

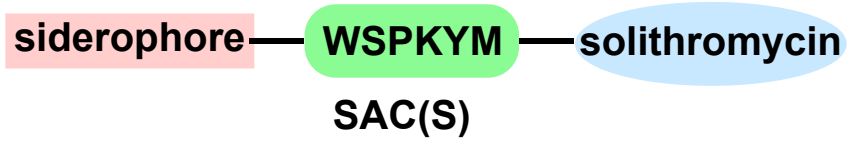
Antibacterial activity against *E. coli MG1665* (wild type) (MIC, μM)

concentration of DP [μM]	0	10	20	40	80	120	160	200	250
SAC(S)	>27	>27	>27	>27	>27	>27	2	0.8	0.8
solithromycin	5	10	5	5	5	5	5	5	5

Antibacterial activity of SACs increased at higher concentration of DP, whereas the activity of controls without siderophore is independent of the concentration of DP.
These results suggest that synthesized SACs are transported by siderophore uptake machinery.

Antibacterial Activity of Solithromycin Conjugates

SAC(S) was used to evaluate the efficiency of linker cleavage because solithromycin itself is active against Gram-negative species.



SAC(S)

Antibacterial activity (MIC, μM)

	solithromycin (parent drug)	L-linker SAC(S) (original SAC)	D-linker SAC(S) (control SAC)
A. nosocomialis	5	7	>27
E. aerogenes	9	7	>27
K. pneumoniae	9	13	>27
E. coli Δ*surA* [a]	1	>27	27
S. aureus Newman [b]	1	>27	>27

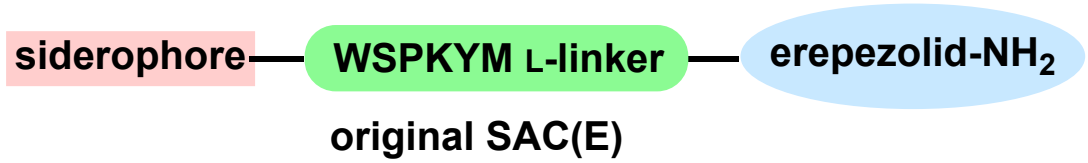
[a] lacking the outer-membrane protein, [b] a Gram-positive specie

In Gram-negative species, L-linker SAC(S) was active as well as solithromycin, but D-linker SAC(S) was less active.

In strains lacking the outer membrane, both L- and D-linker SAC(S) were inactive.

These results suggest that L-linker SAC(S) was cleaved by periplasmic protease as expected and released a solithromycin, which was able to reach its ribosomal target and work well.

Antibacterial Activity of Eperezolid-NH_2 Conjugates



Antibacterial activity (MIC, μM)

difference from original SAC(E)			*E. coli* ΔbamBΔtolC	*E. coli* ΔsurA	*S. aureus* Newman
N-terminus	linker	C-terminus			
	eperezolid-NH_2		>171	43	43
	original SAC(E)		1	38	>38
—	D-linker	—	19	>38	>38
—	—	-OH	48	>48	>48
—	—	-OMe	24	48	>48
—	—	*ent*-eperezolid-NH_2	9	38	>38
—	WSWC	—	37	ND	ND
Ac	—	—	>77	ND	>77

SAC(E) showed potent activity against *E. coli ΔbamBΔtolC*, but inactive against the others.
SAC(E)s without siderophore, cleavable linker or active antibiotic were less active than original one.

By linked with siderophore by cleavable linker, eperezolid-NH_2 was able to reach its ribosomal target in cytoplasm passing through the double membrane barriers.

Antibacterial Activity of Daptomycin Conjugates

original SAC(D)

Antibacterial activity (MIC, μM)

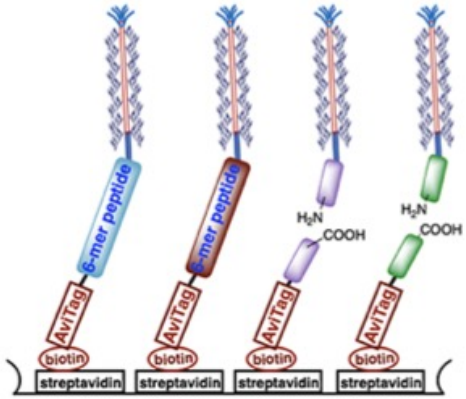
difference from original SAC(D) linker	C-terminus	*E. coli* K12	*E. coli* ΔbamBΔtolC	*A. baumannii*	*A. nosocomialis*	*E. coli* ΔsurA	*S. aureus* Newman
Daptomycin		>39	>39	>39	>39	0.6	0.6
original SAC(D)		11	11	5	1	>21	>21
D-linker	—	>23	23	23	11	23	>23
—	-OH	>48	>48	>24	>48	>48	>48
—	-OMe	>48	24	>24	ND	48	>48

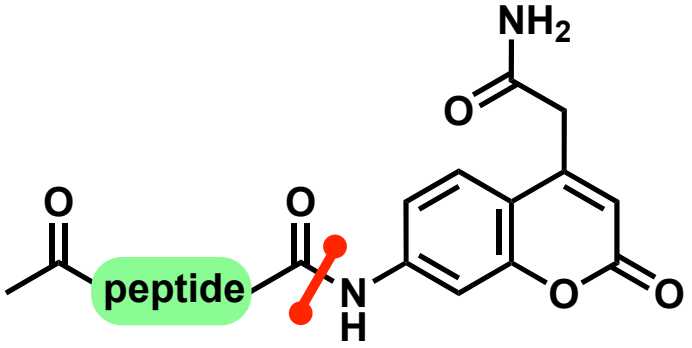
In contrast to daptomycin, L-linker SAC(D) was active against Gram-negative bacteria but inactive against Gram-positive spp.
D-linker SAC(D) was less active against Gram-negative bacteria than L-linker one.

Protease cleavable linker was also beneficial for SACs of periplasmic-targeting drugs.

Summary

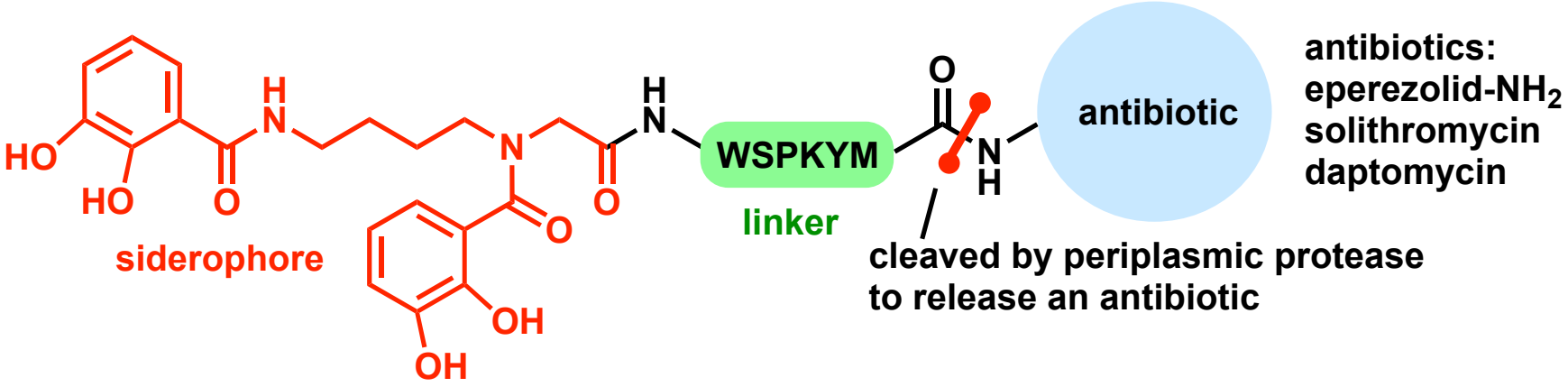
A peptide linker cleavable by bacterial protease was developed by phage display and turn-on fluorescent assay.





cleavage -> release of fluorophore

Three SACs conjugated with cytoplasmic- or periplasmic-targeting drugs were synthesized with the developed cleavable linker.
All of them showed antibacterial activity against Gram-negative bacteria



antibiotics:
eperezolid-NH_2
solithromycin
daptomycin

cleaved by periplasmic protease to release an antibiotic

By using the methodology developed in this study, new prodrugs including SACs will be generated.