Establishment of an Efficient Method for the Synthesis of SRH, an Important Molecule in Bacterial Quorum Sensing

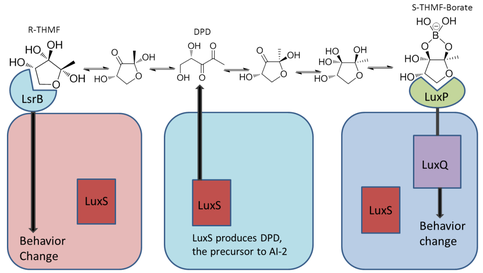
Emily Showell-Rouse, Ruoyi Liu and Megan Bolitho
Department of Chemistry, University of San Francisco

INTRODUCTION

System Two Quorum Sensing (QS)- The process of interspecies bacterial cell-to-cell communication[1]

Secretion and detection of autoinducer-2 → Perception of population density → Synchronized behaviors
- Virulence
- Bioluminescence
- Motility
- Biofilm formation

AI-2 takes on many forms in solution; different forms are detected by different bacterial species[4]



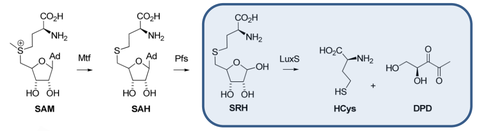
Salmonella typhimurium detects the form of AI-2 called R-THMF

Many different bacteria have been found to produce AI-2

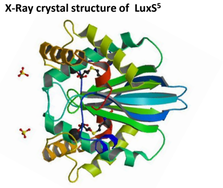
Vibrio harveyi and *Vibrio cholerae* detect the AI-2 form shown above, S-THMF-Borate

Biosynthesis of AI-2 by LuxS

S-ribosylhomocysteine (SRH) is converted to 4(*S*),5-dihydroxy-(2,3)-pentanedione (DPD) and homocysteine (HCys) by *S*-ribosylhomocysteinase (LuxS)[3]

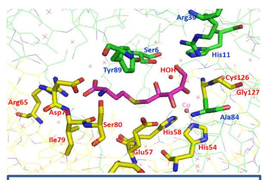


X-Ray crystal structure of LuxS[5]



- Homodimer – 35 kDa
- 2000 Å^2 dimer interface
- Multiple crystal structures solved

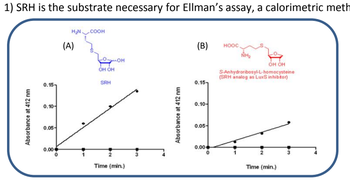
The LuxS Binding Site[5]



monomer A (yellow), monomer B (green), ligand (pink), residues contributed by monomer A (red), residues contributed by monomer B (blue)

Why Synthesize SRH?

1) SRH is the substrate necessary for Ellman's assay, a calorimetric method for detecting LuxS activity.



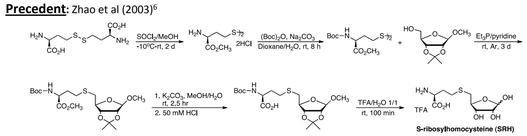
(A) SRH reacts with LuxS showing increased absorbance[7]

(B) SRH analog reacts with LuxS showing decreased absorbance[7]

2) A number of synthetic skills necessary to synthesize SRH are also applicable to the synthesis of SRH analogs as possible LuxS inhibitors.

FIRST ATTEMPT: Unsuccessful at Acquiring SRH

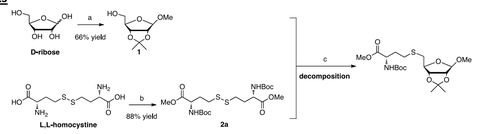
Precedent: Zhao et al (2003)[6]



Scheme 1. **First chemical synthesis of SRH reported by Zhao et al (2003).**

The Key Step - Mitsunobu coupling between the fully protected homocystine and the D-ribose derivative effected the S–C bond formation (the 3rd step above).

Results



Scheme 2. **Unsuccessful attempt of Mitsunobu coupling.**

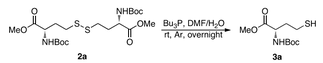
Reaction conditions: a. MeOH/acetone, conc. HCl, reflux, 1.5 h; b. (i) MeOH, $SOCl_2$, 0 °C-rt, Ar, 3 d; (ii) $(Boc)_2O$, dioxane/Na_2CO_3(aq), 0 °C-rt, overnight; c. Bu_3P, pyridine, rt, Ar, 3 d.

Analysis

Possible reasons for the failure of Mitsunobu reaction:

(i) The lack of initial cleavage of the S-S bond in disulfide **2a**

This hypothesis was disproven by the following successful conversion (quantitative yield).



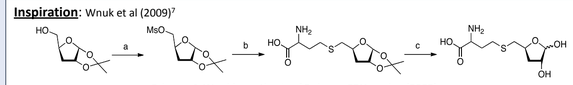
Scheme 3. **Initial step of Mitsunobu reaction – cleavage of the S-S bond**

(ii) Unsuccessful coupling of the homocysteine moiety **3a** to ribose moiety **1** (true reason)

To overcome this problem, better coupling partners, in place of compounds **1** and **3a**, would be required to be utilized in this S_N2 substitution.

SECOND ATTEMPT: Successful Synthesis of SRH without Quantification

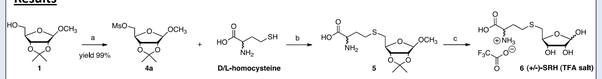
Inspiration: Wnuk et al (2009)[7]



Scheme 4. **Wunk's synthesis of 3-deoxy-SRH analog (2009).**

Reagents: (a) MsCl /Et_3N; (b) HCys/NaOH/MeOH/H_2O; (c) TFA/H_2O.

Results



Scheme 5. **Successful coupling of HCys and activated ribose moiety to produce (+/-)-SRH•TFA salt.**

Reaction conditions: a. MsCl, Et_3N, DCM, 0 °C–rt, Ar, 30 min; b. 1 M NaOH/H_2O, 60 °C, Ar, overnight; c. TFA/H_2O, 0 °C–rt, 3 h.

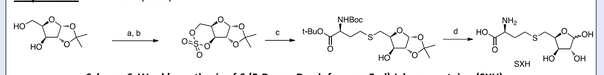
Analysis

Advantages	Disadvantages
1. Utilization of better leaving group 2. Utilization of native HCys instead of protected homocysteine 3. Simpler synthetic pathway	1. Formation of inseparable and undetectable salt byproduct 2. Utilization of aqueous solvent for the coupling reaction

We could not accurately determine the exact amount of (+/-)-SRH produced by this synthetic protocol by weight. This was problematic, as SRH is meant for use in the biochemical assay and needs to be quantified exactly.

THIRD ATTEMPT: Successful Synthesis and Quantification of SRH

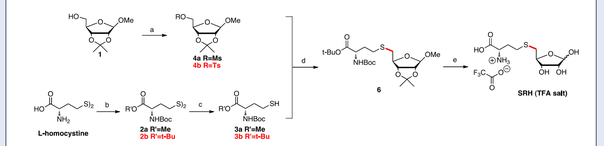
Inspiration: Wunk et al (2009)[7]



Scheme 6. **Wnuk's synthesis of *S*-(5-Deoxy-D-xylofuranos-5-yl)-L-homocysteine (SXH).**

Reagents and conditions: (a) $SOCl_2$/Et_3N/DCM/-78 °C to rt; (b) $RuCl_3$/$NaIO_4$/CCl_4/CH_3CN/H_2O/0 °C to rt; (c) (i) BocNH–CH(CH$_2$CH$_2$SH)–CO$_2$*t*-Bu/BuLi/DMF/0 °C to rt, (ii) THF/H_2SO_4/H_2O/0 °C; (d) TFA/H_2O/0 °C to rt.

Results

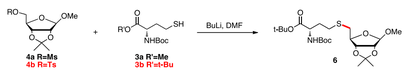


Scheme 7. **Successful hybrid method of SRH synthesis.**

Reaction conditions: (a) TsCl, pyridine, Ar, 0 °C-rt, 1 d, 98%; (b) (i) 10% Na_2CO_3 (aq)/dioxane, $(Boc)_2O$, 0 °C-rt, o/n, (ii) *tert*-butyl-2,2,2-trichloroacetimidate, DCM, Ar, rt, o/n, 90%; (c) Bu_3P, DMF/ H_2O, Ar, rt, o/n, quantitative yield; (d) nBuLi, DMF, Ar, 0 °C–rt, o/n, 70%; (e) TFA/anisole/H_2O, 0 °C–rt, 6 h, 94%.

Analysis

Interestingly, the identity of both the protecting group **R'** on the carboxylate (**3**) and the leaving group **R** on the ribose moiety (**4**) had significant effect on the success of coupling reaction (S-C bond formation).



	Mesylate (Ms) 4a	Tosylate (Ts) 4b
Methyl (Me) **3a**	✗	✗
Tert-butyl (tBu) **3b**	✗	✓

Scheme 8. **Coupling results of combinations of protecting groups and leaving groups.**

Advantages (comparing with the second attempt):

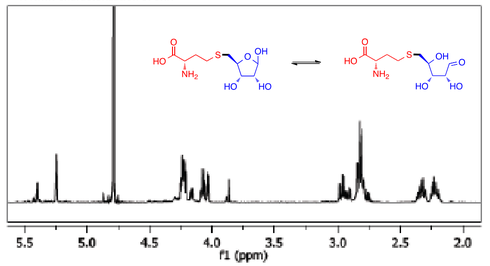
(i) The obtained TFA salt of SRH is a pure, powdery solid (after lyophilization) that could be easily measured by weight.

(ii) The use of enantiomerically pure materials produced the single desired isomer of SRH in very good overall yield (~60% over five linear steps).

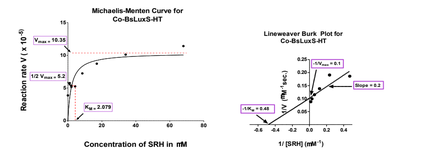
CHEMICAL CHARACTERIZATION

Synthetic intermediates and products were largely characterized using nuclear resonance spectroscopy (NMR).

^{1}H NMR Characterization of SRH



BIOLOGICAL CHARACTERIZATION



Enzyme	V_{max} (μM/sec.)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
Co-BsLuxS-HT	10.35±1.480	2.079±1.500	12.95±0.168	622.9×10^4
Co-BsLuxS-HT	Not provided	2.3±0.5	0.035±0.003	1.6×10^4

(C)

Michaelis-Menten curve, Lineweaver Burk Plot and Comparison of Kinetic Constants. (A) Michaelis-Menten curve for Co-BsLuxS-HT purified in our lab conditions. Red dotted lines show the values of kinetic constants on the curve. (B)Lineweaver Burk Plot for calculation of kinetic parameters (C) Kinetic constants of the two Co-BsLuxS-HT enzymes. The K_M values for both the enzymes are almost same confirming that our enzyme is equally active.

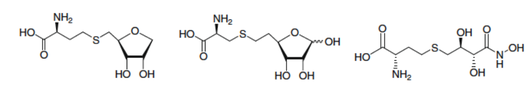
CONCLUSIONS

- We reported our three attempts to synthesize SRH and finally achieved the best synthetic protocol towards SRH synthesis.
- SRH•TFA salt was obtained as the pure solid that is ready for use in the biochemical assay.
- The information obtained after extensive troubleshooting from synthesis of SRH could be used to inform synthetic pathways towards SRH analogs.
  - A. Both protecting groups on the homocysteine moiety and leaving groups on the ribose moiety have great effect on the Mitsunobu coupling reaction.
  - B. In order to obtain the quantified SRH product with exact weight, organic solvents must be utilized in the coupling reaction.

FUTURE DIRECTION: Synthesis of SRH analogs

SRH analogs may serve as potential inhibitors of LuxS

- Reported SRH analogs by Zhou et al[8] (focus on the modification of ribose part)



Our designed SRH analog type (focus on the modification of homocysteine part)

- General idea toward the synthesis of this type of molecule:

(i) the synthesis of the modified homocysteine moiety containing the novel "R" group at the C3 position*.

(ii) the coupling of the amino acid moiety with the ribose moiety.

Cyclic and ring-opened forms of our proposed SRH analogs

R_1 = alkyl, aryl; R_2 = H
R_1 = H; R_2 = alkyl, aryl
R_1, R_2 = alkyl, aryl

REFERENCES

1. Waters, C. M.; Bassler, B. L. *Ann. Rev. Cell. Dev. Biol.* **2005,** *21*, 319.
2. Xavier, K. B.; Bassler, B. L. *Curr. Opin. Microbiol.* **2003,** *6*, 191.
3. Schauder, S. G. et al. *Mol. Microbiol.* **2001,** *41*, 463.
4. Miller, S. T. et al. *Mol. Cell.* **2004,** *15*, 677.
5. Rajan, R. et al. *Biochemistry.* **2005,** *44*, 3745.
6. Zhao, G. *Bioorganic & Medicinal Chemistry Letters.* **2003,** *13* (22), 3897.
7. Wunk S. F. et al. *Bioorg. Med. Chem.* **2009,** *17*, 6699.
8. Alfaro, J.F. et al. *Org. Lett.* **2004**, 6, 3043.
9. Zhu, J. et al. *Biochem.* **2003**, *42*, 4717.

ACKNOWLEDGEMENTS

- This work is supported by the USF College of Arts & Sciences and the USF Faculty Development Fund.
- Thanks for Jeff Oda, Andy Huang, Angela Qin and Chad Schwietert's support for utilizing facilities.