Late-Stage Nitration of Amanitin Analogues for Hypoxia-Activated Prodrugs

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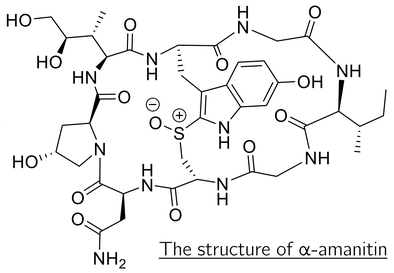
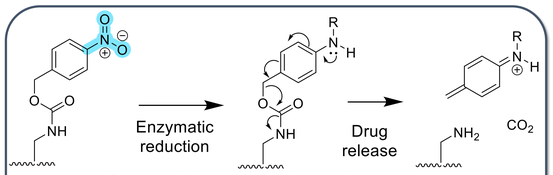
Introduction

Alpha amanitin is the principal toxin of the *Amanita* mushroom genus and one of the deadliest natural products [1]. While α-amanitin is of interest as a therapeutic agent, its high off-target toxicity must be reduced, such as through improving drug selectivity using prodrug strategies.

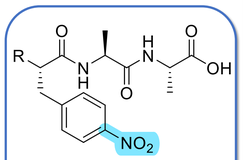


The structure of α-amanitin

Nitroaryl moieties in peptides are appealing for their interactions in physiological systems. Hypoxia, the insufficient levels of oxygen for cells, is a common tumour microenvironment and leads to the upregulation of nitroreductase enzymes. Aromatic nitro groups are reduced by these enzymes and can be used as a trigger for drug release in hypoxia-activated prodrugs [2]. As well, amino acids with nitroaromatic regions have been shown to be highly immunogenic [3]. A nitrated amanitin analogue may be selectively activated in cancer cells.

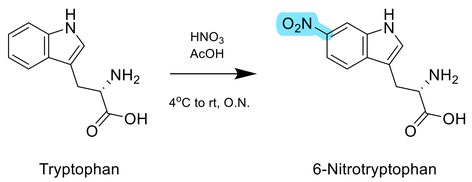


One possible nitro-prodrug release mechanism [2]



C-terminal of nitrated T cell epitope [4]

However, the current 'mixed acid' approach to nitrating amino acids like tryptophan is performed under harsh reaction conditions, leading to nonselective nitration, oxidized side products and poor functional group tolerance [5].

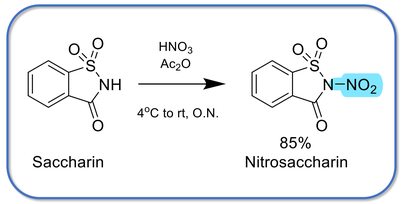


Tryptophan — 6-Nitrotryptophan

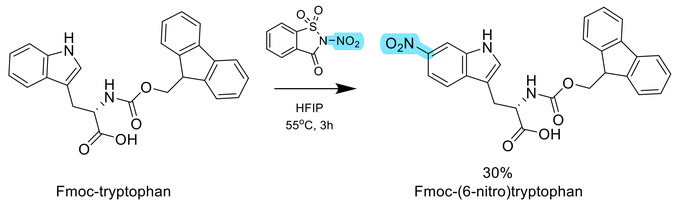
The 'mixed acid' approach to nitrotryptophan synthesis

An Alternative Nitrating Agent: Nitrosaccharin

Nitrosaccharin is a bench-stable nitrating agent capable of nitrating a wide array of aromatic compounds [5]. Using nitrosaccharin, Fmoc-protected tryptophan was successfully nitrated selectively at the C6 of the indole.



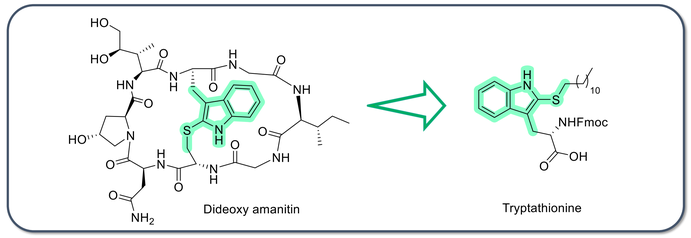
Saccharin — Nitrosaccharin



Fmoc-tryptophan — Fmoc-(6-nitro)tryptophan

Nitration of the Amanitin Tryptathionine

Alpha-amanitin and its active analogues contain an aromatic tryptathionine staple; nitration strategies towards nitrated amanitins were tested on a mimic of this residue.

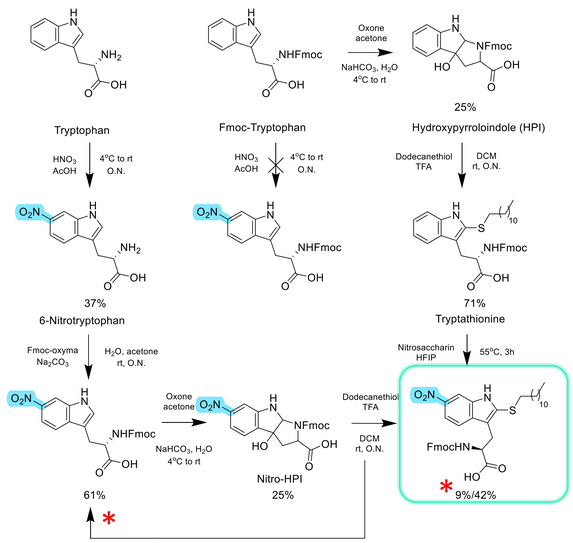


Dideoxy amanitin — Tryptathionine

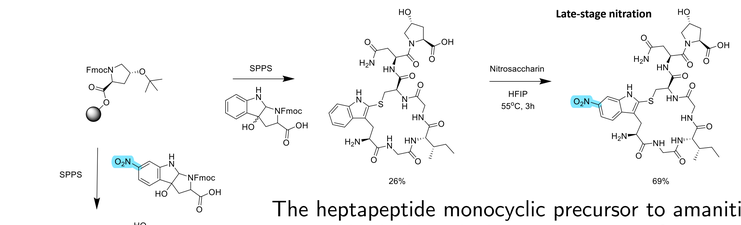
Strategies toward the synthesis of nitro-tryptathionine

The tryptathionine mimic was nitrated first by oxidizing nitrotryptophan to HPI; however, the subsequent tryptathionylation was low yielding.

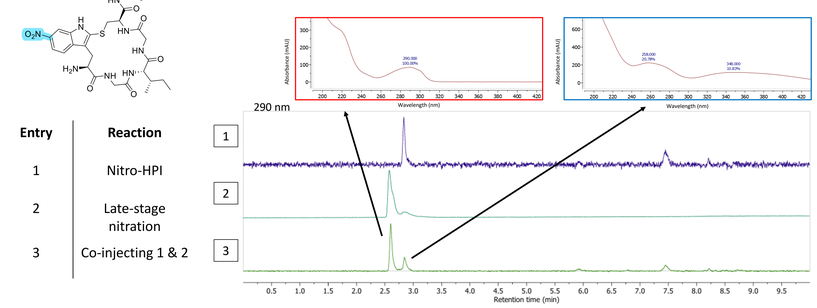
By nitrating tryptathionine with nitrosaccharin, nitrotryptathionine was synthesized in improved yields.



Two routes to nitrated heptapeptide synthesis



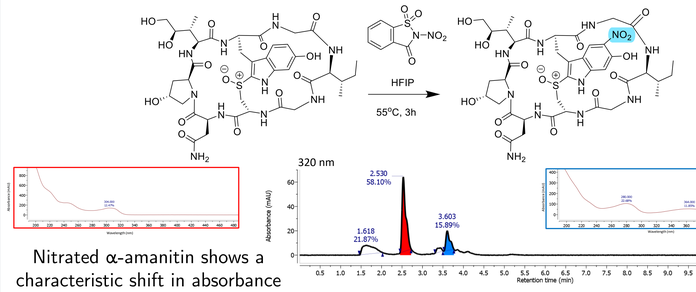
The heptapeptide monocyclic precursor to amanitin was nitrated both through the incorporation of nitro-HPI and through the late-stage nitration with nitrosaccharin.



Entry	Reaction
1	Nitro-HPI
2	Late-stage nitration
3	Co-injecting 1 & 2

Co-injecting the nitrated heptapeptides from both routes confirmed the same product: 6-nitrotrypthionine stapled heptapeptide, with a characteristic absorbance profile.

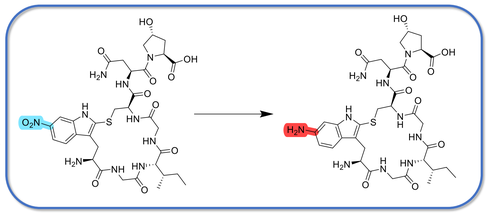
Late-Stage Nitration of α-Amanitin

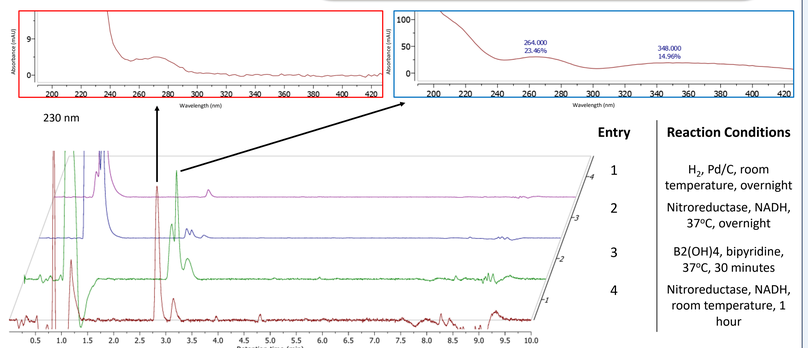


Nitrated α-amanitin shows a characteristic shift in absorbance

Reductions of Nitrated Heptapeptide

The nitrated heptapeptide was reduced under chemical and enzymatic conditions to confirm nitroreductase susceptibility of the prodrug analogues.





Entry	Reaction Conditions
1	H_2, Pd/C, room temperature, overnight
2	Nitroreductase, NADH, 37°C, overnight
3	B2(OH)4, bipyridine, 37°C, 30 minutes
4	Nitroreductase, NADH, room temperature, 1 hour

Compared to a reduced standard (entry 1), the nitrated heptapeptide was reduced under enzymatic and biocompatible chemical conditions.

Conclusions and Future Directions

The heptapeptide precursor to amanitin was selectively and efficiently nitrated using nitrosaccharin as a nitrating agent. The mild late-stage nitrating capabilities of nitrosaccharin also allowed for direct modification on α-amanitin. With the nitroreductase enzyme confirmed to reduce the nitrated heptapeptide, the nitrated amanitin analogues will be carried forward into cell viability assays under normoxic and hypoxic conditions to determine their prodrug capabilities.

Acknowledgements

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References: 1. Matinkhoo, K. *et al.* Design, Synthesis, and Biochemical Evaluation of Alpha-Amanitin Derivatives Containing Analogs of the trans-Hydroxyproline Residue for Potential Use in Antibody-Drug Conjugates. *Chemistry – A European Journal* **27**, 10282–10292 (2021). 2. Denny, W. A. Nitroaromatic Hypoxia-Activated Prodrugs for Cancer Therapy. *Pharmaceuticals* **15**, 187 (2022). 3. Grünewald, J. *et al.* Mechanistic studies of the immunochemical termination of self-tolerance with unnatural amino acids. *Proceedings of the National Academy of Sciences* **106**, 4337–4342 (2009). 4. Tian, H. *et al.* Nitrated T helper cell epitopes enhance the immunogenicity of HER2 vaccine and induce anti-tumor immunity. *Cancer Letters* **430**, 79–87 (2018). 5. Calvo, R., Zhang, K., Passera, A. & Katayev, D. Facile access to nitroarenes and nitroheteroarenes using N-nitrosaccharin. *Nat Commun* **10**, 3410 (2019).