Efficient and glycoeconomic synthesis of densely clustered mucin glycopeptides

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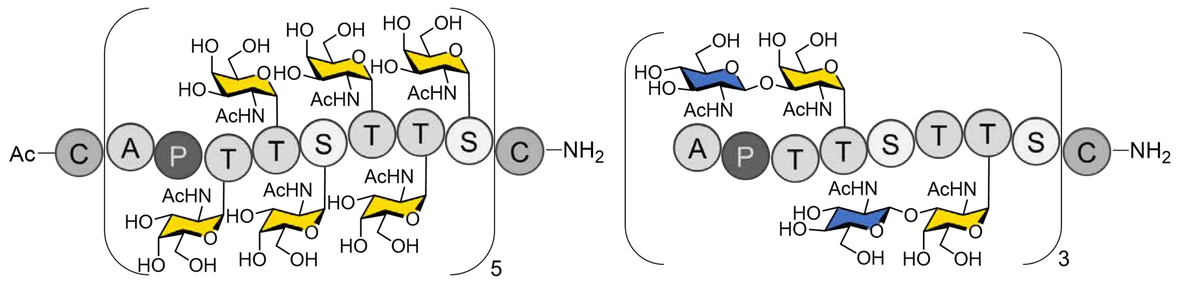
Introduction

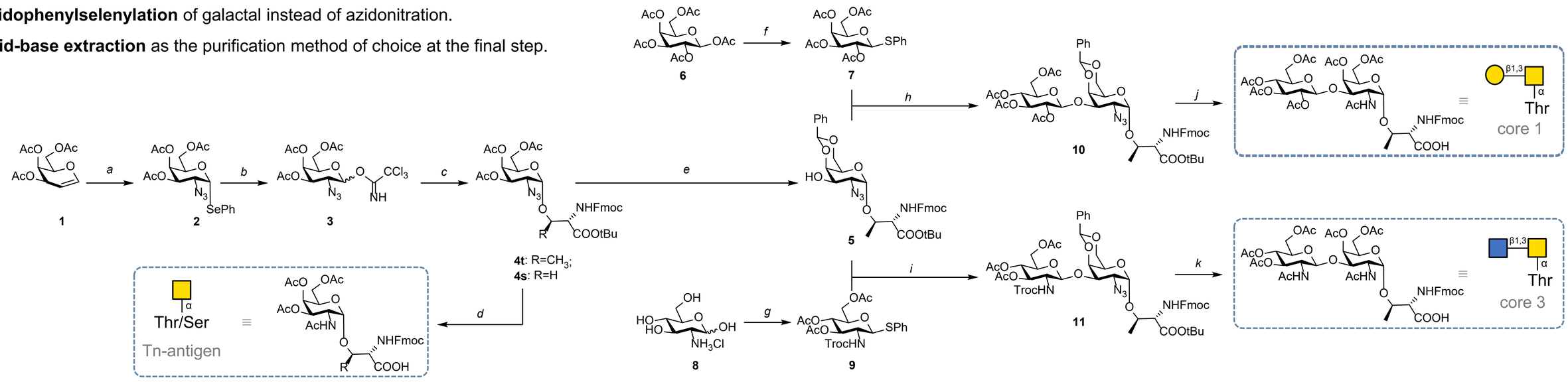
Mucins are known for fully O-glycosylated multiple tandem repeats (TRs), like the PTTSTTS motif found in MUC5AC, which has ~240 copies and 6 glycosylation sites per TR. The lack of methods to access long, glyco-defined multi-TRs limits our understanding of how mucin glycosylation impacts its functional properties. While solid-phase synthesis (SPS) is a viable approach, its disadvantage is the requirement of excess amounts of costly and potentially delicate glycoamino acids. We strive to improve access to glycoamino acid building blocks and develop methods allowing glycoeconomic synthesis of densely clustered mucin glycopeptides.



Chemical structures of exemplar synthesized glycopeptides.

Multigram synthesis of mono[1]- and di-glycosylated building blocks

- ✓ **Azidophenylselenylation** of galactal instead of azidonitration.
- ✓ **Acid-base extraction** as the purification method of choice at the final step.



Conditions. (a) Se_2Ph_2, $TMSN_3$, (diacetoxyiodo)benzene, CH_2Cl_2, -30°C (55%); (b) i. NIS, acetone, H_2O, RT (quant.); ii. CCl_3CN, Cs_2CO_3, CH_2Cl_2, RT (84%); (c) Fmoc-Thr/Ser(OH)-*t*Bu, TMSOTf, CH_2Cl_2, Et_2O, 0°C; (d) i. Zn, THF, AcOH, Ac_2O, RT (47-51% for 2 steps); ii. TFA, CH_2Cl_2, RT (95-97%); (e) i. 1M NaOMe, MeOH; ii. $PhCH(OMe)_2$, CSA, CH_3CN (82% for 2 steps); (f) $BF_3{\cdot}Et_2O$, PhSH, CH_2Cl_2 (83%); (g) i. TrocCl, $NaHCO_3$, H_2O; ii. Ac_2O, Py (50% for 2 steps); iii. $BF_3{\cdot}Et_2O$, PhSH, CH_2Cl_2 (84%); (h) NIS, TMSOTf, CH_2Cl_2 (55%); (i) NIS, TMSOTf, CH_2Cl_2 (76%); (j) i. CSA, DTT, CH_2Cl_2; ii. Zn, AcOH, THF; iii. Ac_2O, Py (70% for 3 steps); iv. anisole, TFA (97%); (k) i. 80% AcOH, 80°C; ii. Zn, AcOH, THF; iii. Ac_2O, Py (64% for 3 steps); iv. anisole, TFA (91%).

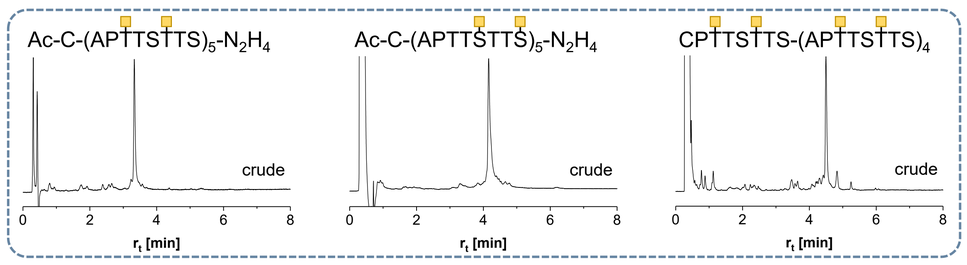
Solid-Phase Synthesis of mucin glycopeptides

Semi-automated synthesis of partially monoglycosylated MUC5AC peptides[1]

- ✓ Development of fast and clean coupling of Fmoc-Thr/Ser(αAc_3GalNAc) with only **1.5 eq**.
  → DIC/Oxyma in **2-MeTHF** instead of DMF, **10 min** coupling time, **no microwave**.
- x Due to low viscosity of 2-MeTHF, coupling of glycoamino acid must be performed manually.

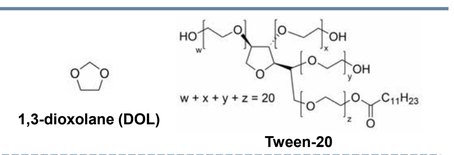
Peptide	Fmoc deprotection	Coupling	Capping	Sugar deprotection	Yield[a]
Ac-C-(APTTSTTS)$_5$-N_2H_4	20% Piperidine in DMF	AA: 5 eq. AA/HATU/Oxyma, 15 eq. DiPEA in DMF; **GlycoAA**: 1.5 eq. AA/DIC/Oxyma in 2-MeTHF	Ac_2O/ DiPEA in DMF	$N_2H_4{\cdot}H_2O$ in DMF (50:50)	17%
Ac-C-(APTTSTTS)$_5$-N_2H_4					9%
CPTTSTTS-(APTTSTTS)$_4$					9%

[a] *Yields calculated after HPLC purification*



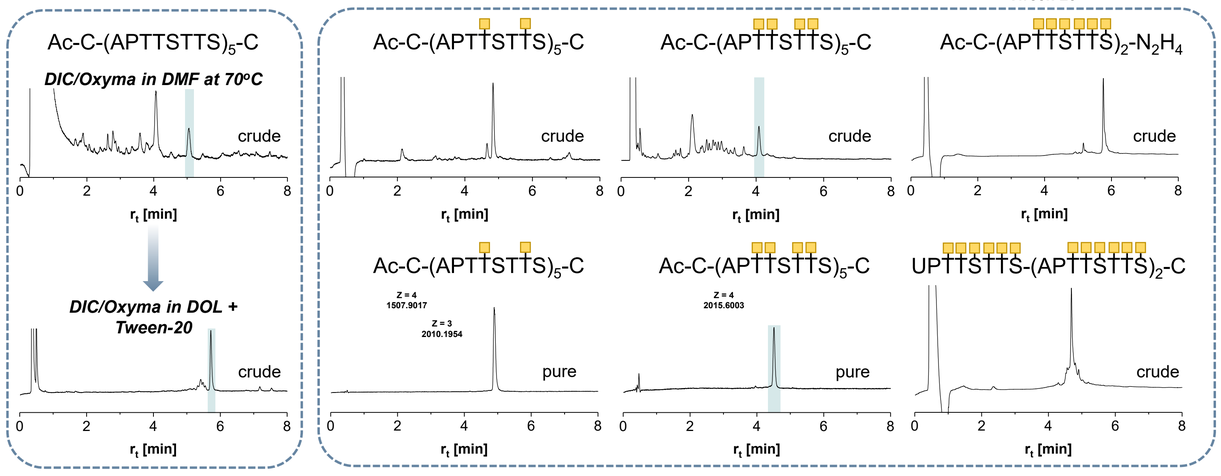
Automated synthesis of partially and fully monoglycosylated MUC5AC peptides

- ✓ Automated DMF-free synthesis with green solvent **1,3-dioxolane (DOL)/Tween-20** furnishes high yields of isolated material.
- ✓ **Tween-20** is added with the intention to prevent on-resin aggregation, allowing the synthesis of difficult MUC5AC peptide.
- X However, synthesis of highly glycosylated peptides remains challenging.



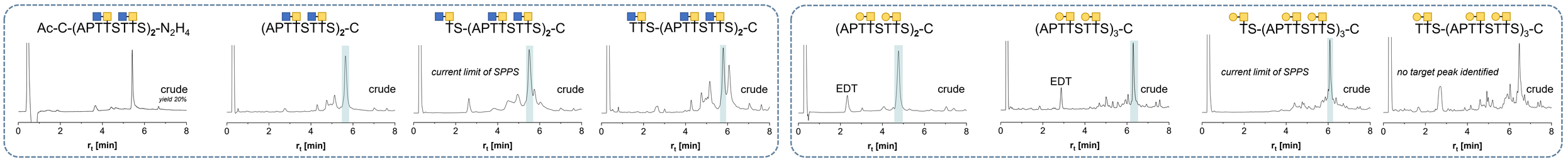
Peptide	Fmoc deprotection[a]	Coupling[a]	Capping	Sugar deprotection	Yield[b]
Ac-C-(APTTSTTS)$_5$-C	20% Pyrrolidine in DOL	1.5 eq. AA/DIC/Oxyma in DOL	Ac_2O/ DiPEA in DOL	$N_2H_4{\cdot}H_2O$ in DMF (50:50)	18%
Ac-C-(APTTSTTS)$_5$-C					16%
Ac-C-(APTTSTTS)$_5$-C					2%
UPTTSTTS-(APTTSTTS)$_2$-C					5%
Ac-C-(APTTSTTS)$_2$-N_2H_4					27%

[a] *1% Tween-20 (v/v) was added to all synthetic mixtures;* [b] *Isolated yields calculated after HPLC purification*



Manual synthesis of partially diglycosylated MUC5AC peptides

- X Core-3 diglycosylated peptides with only two glycoamino acids per TR **behave similarly** to fully monoglycosylated peptides during SPS.
- ✓ Changing GlcNAc to Gal in the disaccharide (core-3 → core-1) **enables the elongation** of peptides up from 2 TRs to 3 TRs.



* *synthesis was performed manually with the same conditions as stated above*

Ligation techniques to obtain long mucin glycopeptides

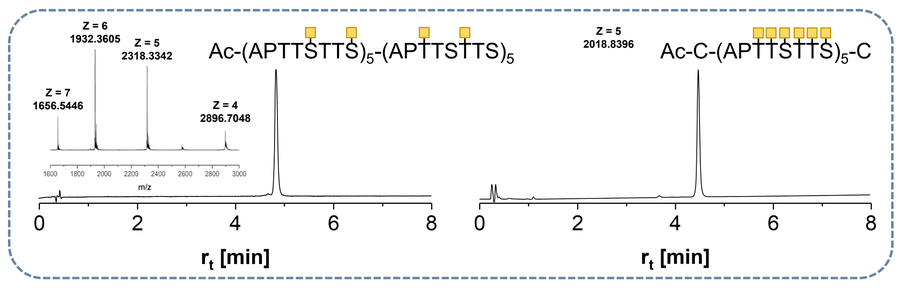
- ✓ C-terminal glyco-serine **does not interfere** with either ligation or dechalcogenation methods

Native Chemical Ligation with C-terminal glycoamino acid[1]

Ac-(APTTSTTS)$_5$-NH-NH_2 → (1. MPAA, acac, Gd-HCl buffer; 2. MPAA extraction with EtOAc, then $NaBEt_4$/TCEP; CPTTSTTS-(APTTSTTS)$_4$) → Ac-(APTTSTTS)$_5$-(APTTSTTS)$_5$ with 80 aa, 20 GalNAc, yield 17%

Diselenide-Selenoester Ligation with C-terminal glycoamino acid

Ac-C-(APTTSTTS)$_2$-NH-NH_2 → (1. Ph_2Se_2, TCEP, acac, buffer A, pH 3, 37°C; 2. Gd-HCl buffer, pH 7, RT; 3. Ph_2Se_2 extraction with hexane, then TCEP/DTT; (UPTTSTTS-(APTTSTTS)$_2$-C)$_2$) → Ac-C-(APTTSTTS)$_5$-C with 42 aa, 30 GalNAc, yield 22%



Conclusions

- ✓ Replacing azidonitration of galactal with azidophenylselenylation facilitated multi-gram scale (16 g) access to glycoamino acids.
- ✓ Employment of DOL/Tween-20 mixture allows DMF-free synthesis of fully glycosylated mucin-like peptides with high purity.
- ✓ Highly monoglycosylated peptides are difficult to access by SPS. Diglycosylated peptides are especially hard to extend.
- ✓ Core-3 (GlcNAcβ(1→3)GalNAc) glycopeptides are more challenging to extend than core-1 (Galβ(1→3)GalNAc) glycopeptides.
- ✓ High glycosylation degree and glycosylation at the ligation junction do not interfere with ligation and dechalcogenation.

References

[1] Galashov, A., Kazakova, E., Stieger, C. E., Hackenberger, C. P. R., Seitz, O. (2024). *Chemical Science, 15*(4), 1297-1305.

Acknowledgements

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