Advances in Peptide Synthesis: Sustainable Approaches through Green Chemistry

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Why Green Chemistry?

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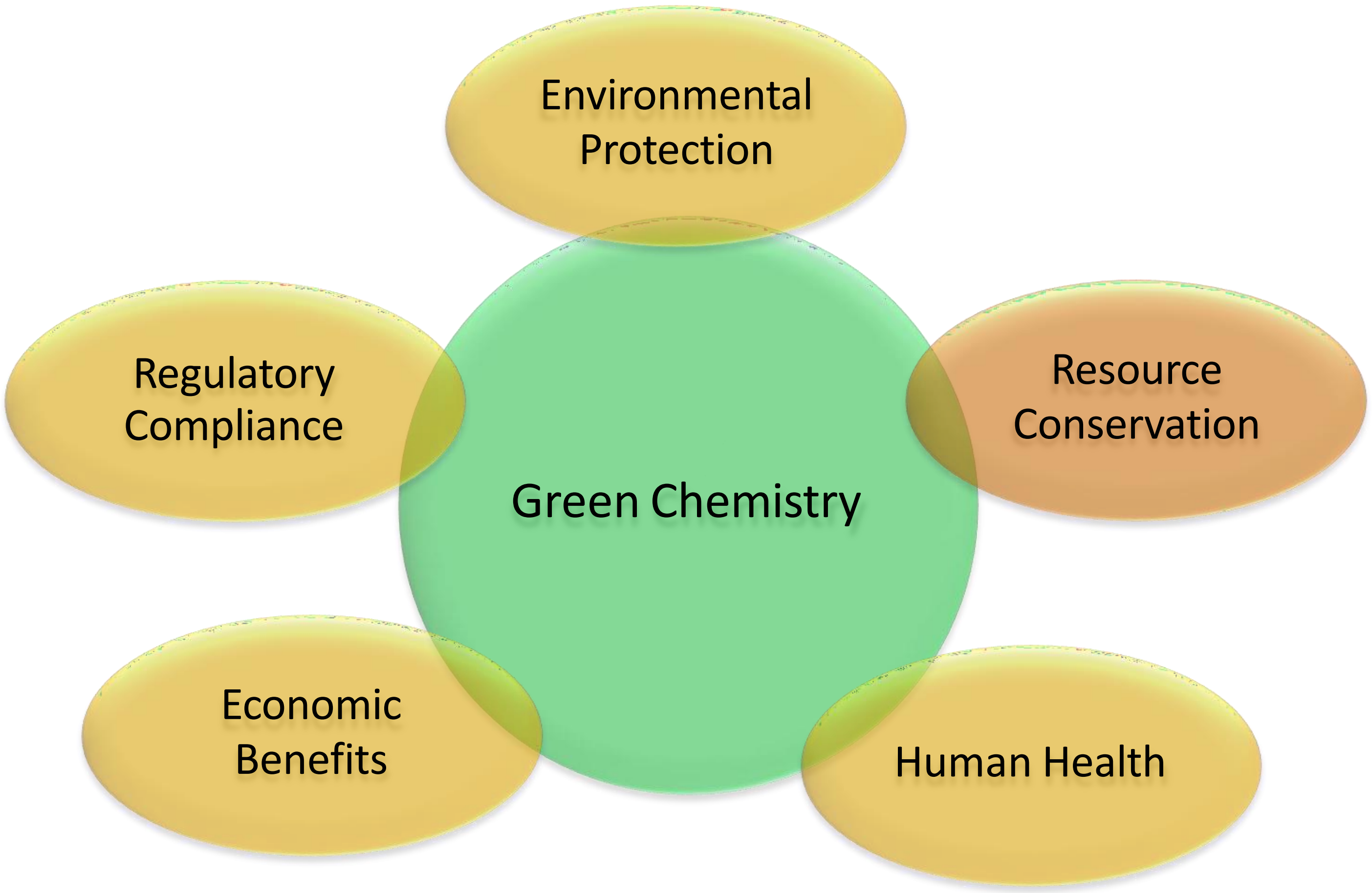

REACH: The European Chemicals Agency (ECHA)

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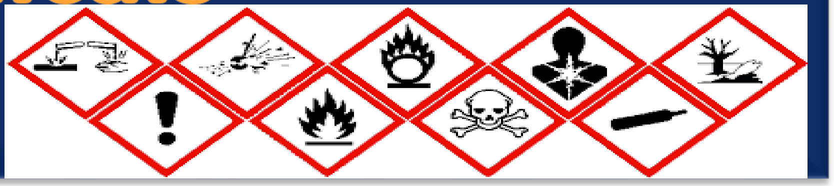
REACH: Registration Evolution Authorization and Restriction of Chemicals



REACH is the main EU law to protect human health and the environment from the risks that can be posed by chemicals.

From December 2023 DMF, DCM are restricted for industrial applications

END GOAL: Better protection from negative health effects on workers such as liver damage, GI toxicity etc.

Principles of Green Chemistry

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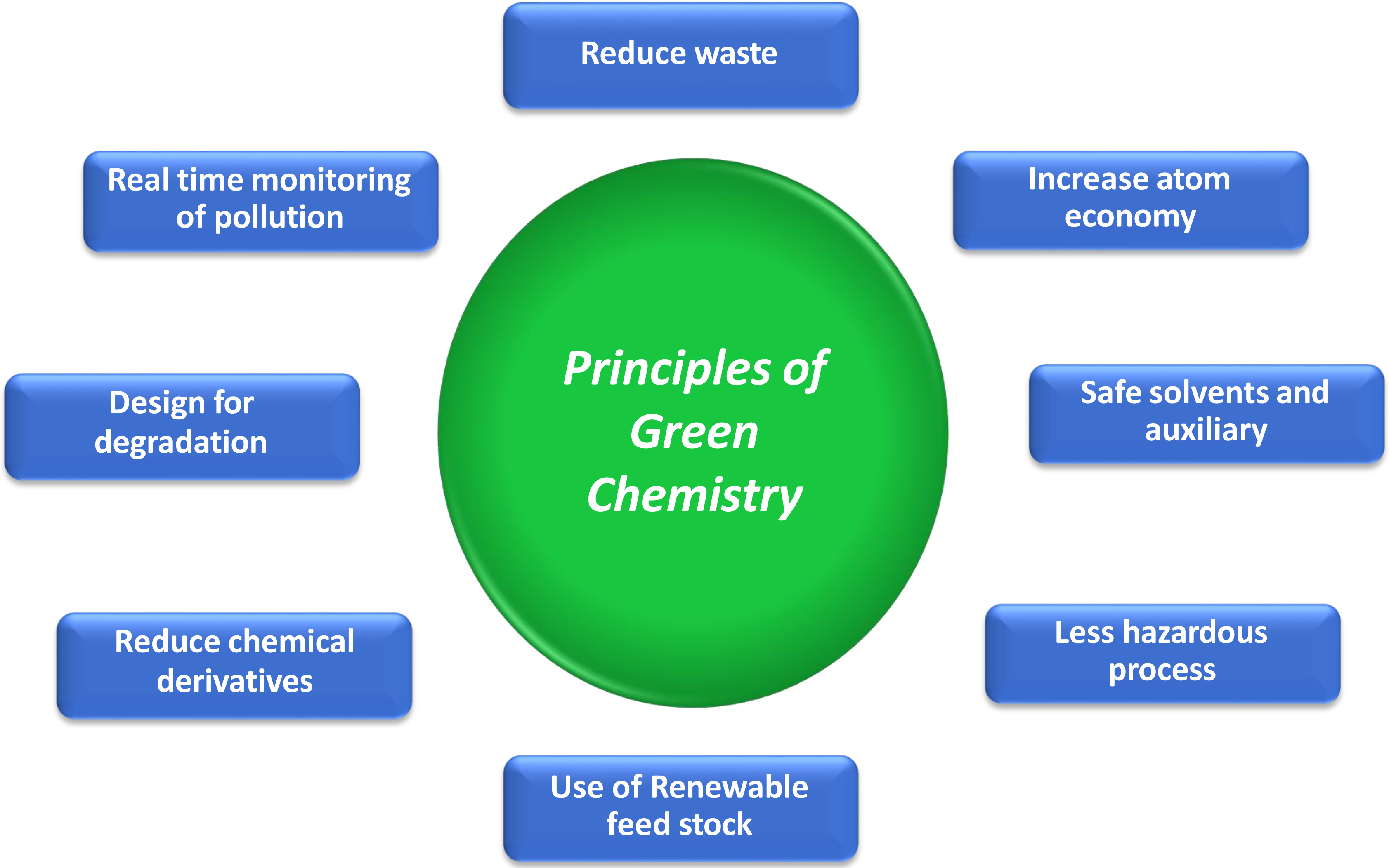

Chemistry for Green SPPS: Need of The Future

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Large solvent use	• Negative environmental footprint • Most of the solvents in SPPS are environmentally hazardous and needs to be replaced
Poor Atom Economy	• Excess of Reagents used for maximum couplings • Poor content, Excess of impurities and byproducts
Current regulation by REACH*	• Classic SPPS solvents DMF, DCM, NMP, DMAc heading for restriction for use

*Registration, Evaluation, Authorisation and Restriction of Chemicals

Pros and Cons of SPPS

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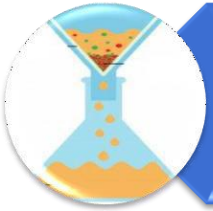
Pros

Single pot reactions no major cleaning procedures, No intermediates isolation

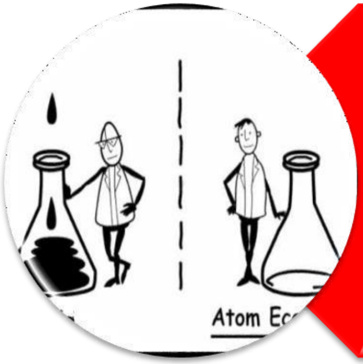
Use of high boiling point solvents, Recycling possible

High efficiency and reduced labour hours



Excess reactants and soluble side products removed by filtration and washing

Cons



High mol wt of KSMs yield low atom economy

Large excesses of reagents required for good yields

Green Chemistry approach addresses conventional challenges

Qualification Criteria for Solvents In SPPS

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Viscosity and melting point	• Viscosity of ≤ 4 mPa*s viscosity nearer that of DMF (0.8 mPa*s) • M.P. >10°C
Scalability	• Dissolution of Reactant and by-products • Solubility ≥0.25 M (ideally up to 0.40 M) • Good stability for at least one week at room temperature
Resin swelling	• Swelling range of approximately 4–7 ml/gm
Process performance	• Loading >1 mmol/g for short chain & > 0.30 mmol/g for long chain. • The reaction time at room temperature should NMT 120 min and below 30–40 min for Fmoc-removal

GVL: Greener and Suitable Alternative to DMF

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Properties	DMF	GVL
Density (g/mL)	0.95	1.05
Flash point (°C)	58	96
Viscosity (CP, 25 °C)	0.92	1.86
Melting point (°C)	-31	-31
Boiling point (°C)	153	207

γ-Valerolactone (GVL) is a renewable, low-toxic molecule obtained from lignocellulosic biomass, and it is therefore non-toxic and biodegradable which presents interesting properties for usage as a solvent, supporting the development of sustainable and safe processes.

Reference : Dunn P.J. The importance of green chemistry in process research and development. Chem. Soc. Rev. 2012;41:1452–1461. doi: 10.1039/C1CS15041C.

Green Chemistry for SPPS: Changes Implement

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Use of solvents like	• GVL, NFM, Ethyl Acetate, Acetone
Mix solvent strategy	• Green solvent + conventional
Effective coupling reagents	• T3P®, T-Bec® to increase productivity
Change in de-protection strategy	• DBU/GVL & use of 1% Oxyma in DMF for washing
Purification solvents	• Use of Alcohols for purification of Peptides
Recycling	• Recycling of Solvents & resins

Case Study

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- Compatibility with existing methodologies
- Cost considerations
- Supply chain of green reagents

The integration of green chemistry principles offers promising opportunities to advance peptide synthesis towards a greener and more sustainable future.

Conventional Protocol for SPPS

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Synthesis	• Sequential coupling of Fmoc-Amino Acid using DIC-HOBt in **DMF** • Washing after coupling **DMF**
Deprotection	• Removal of Fmoc- using **Piperidine in DMF** • Washing with **DMF** and IPA after Fmoc-removal
Cleavage	• Cleavage & side chain de-protection using **TFA** and precipitation require excess amount of **Ether**
Oxidation	• Oxidation or S-S bond formation at lower concentration < 1.0 mg/ml
Purification	• RP Purification using Acetonitrile followed by lyophilisation

Synthesis of Octreotide Green- SPPS

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Synthesis

- Coupling reagent to increase coupling efficiency **T3P** & **Oxyma**
- Use of **GVL (γ- Valerolactone)** for coupling and other process
- Use of mix solvents strategy for process

Deprotection

- Use of **4-Methy Piperidine/GVL, DBU/GVL** for de-protection process

Cleavage

- Cleavage & side chain de-protection using TFA and cold **water,** flammable Ether are completely eliminated

Oxidation

• Oxidation o r S-S bond formation at lowe r concentration < 1.0 mg/ml

- Oxidation of S-S bond formation at lower concentration < 1.0 mg/ml, Dissolved in **water**

Purification

- Use of **Ethanol** or **Methanol** for RP Purification

Green -SPPS: Development Experiments

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Synthesis of Peptidyl resin using GVL as coupling solvents with input Resin H-Thr(tBu)-OL-CTC

	Conventional	Expt. - I	Expt. - II	Expt. – III
Strategy	Conventional synthesis using AA: HOBt : DIC (1.5:2:2) **DMF** as solvent 10V	Coupling using **T3P**, (6 equi.), **Oxyma** (6 equi.) , DIEA (6 equi.) & **GVL** 5V as coupling solvent Washing solvent volume reduce to **50%** as compare to conventional	Coupling using **T3P**, (6 equi.), **Oxyma** (6 equi.) , DIEA (6 equi.) & **GVL** 5V as coupling solvent **No washings after coupling.** After De-protection IPA wash, 2 washings of 1% Oxyma in DMF.	Coupling using AA: HOBt. : DIC (1.5:2:2) & **GVL** 5V as solvent. **No washings after coupling** Fmoc-Deprotection using **2%DBU** & **2% Piperidine in GVL** After de-protection IPA wash, 2 washings of HOBt in DMF
% Yield of Synthesis	90 to 100 %	100%	87.1 %	100 %

Note: %Yield calculations are based on functionality provided by commercial supplier of resin

Green-SPPS: Development Experiments

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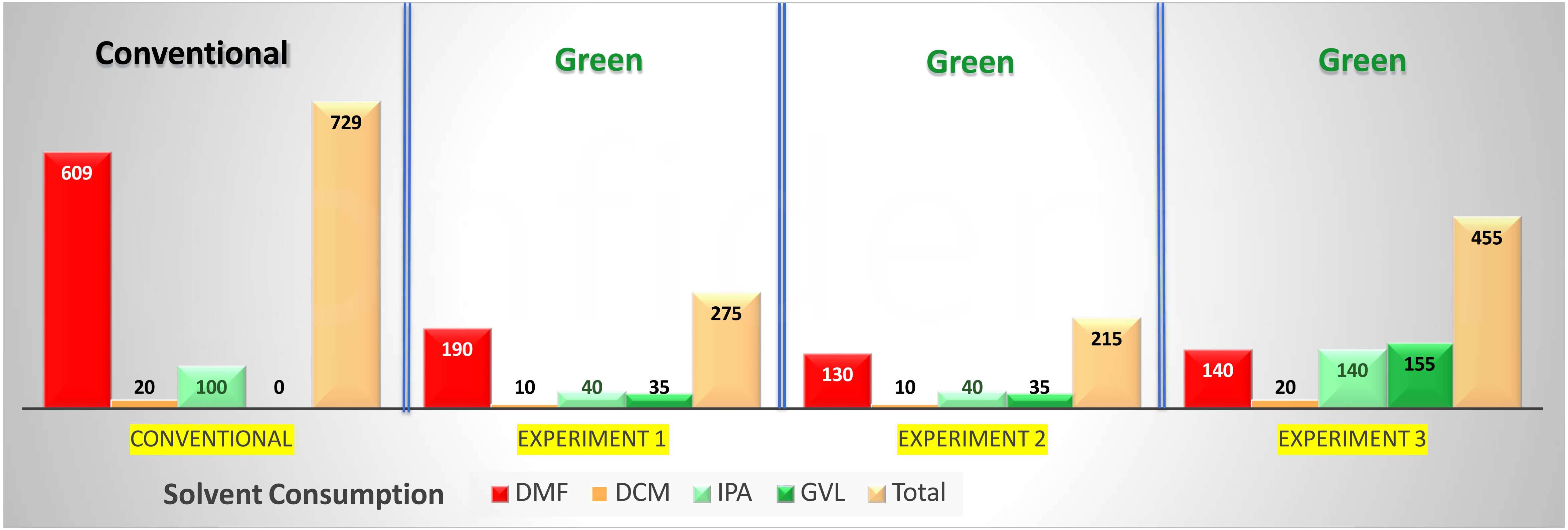
Comparative solvent consumption in V/W* of resin during synthesis of peptidyl resin, conventional Vs Green approach



*All values are based on V/w of resin, e.g. for initial weight of 10 gm resin 10 volume will be 100 ml

Green-SPPS: Development Experiments

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Greener approach in cleavage and oxidation process

Sr. No.	Experiment	Cleavage condition	Oxidation
1	Conventional Approach	• Swell with 6V DCM • 6V cocktail TFA:TIPS:DODT (90:5:5), 3Hr RT • Filter resin, distillation, cooled 10 to 15C • Precipitate with 6V **Di-isopropyl Ether**, • Filter & Wash with MTBE.	• Crude dissolve in water 1mg/ml pH 2.2 • Charge Iodine 10% iodine • Stir for 3Hrs, quench with 0.1M ascorbic acid • Filter RM
2	Experiment I-A		
3	Experiment I-B	• Swell with 6V DCM • 6V cocktail TFA : TIPS : **Water** (90:5:5), 3Hr RT • Filter resin, distillation, cooled 10 to 15C • Dissolved in 100V **cold water**	• Charge Iodine 10 % iodine • Stir for 3Hrs, quench with 0.1M ascorbic acid • pH adjusted with 10N NaOH. to pH 6.0 • Filter RM, 1micron whatman,1.2,0.45 micron filter

Green-SPPS: Development Experiments

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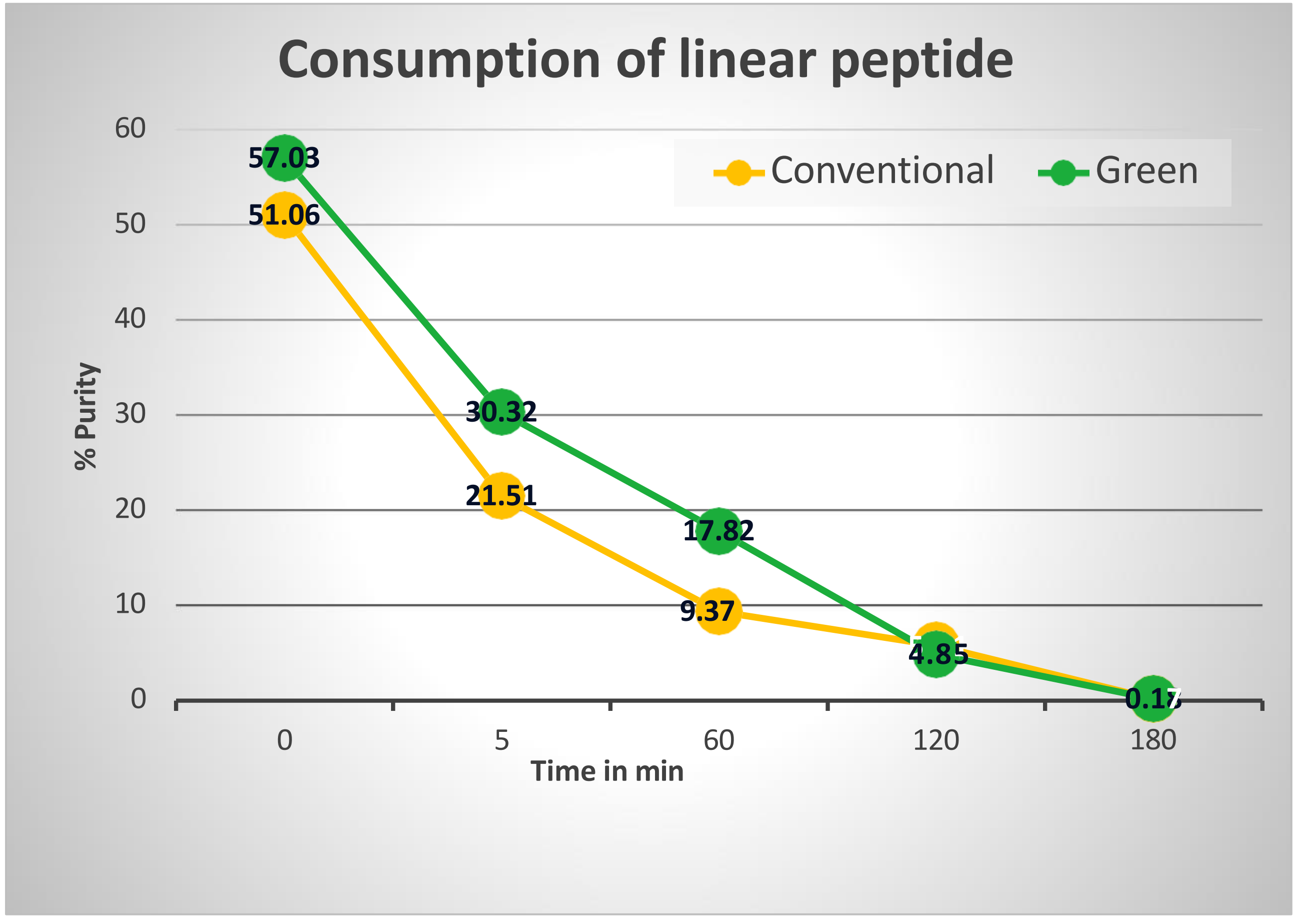
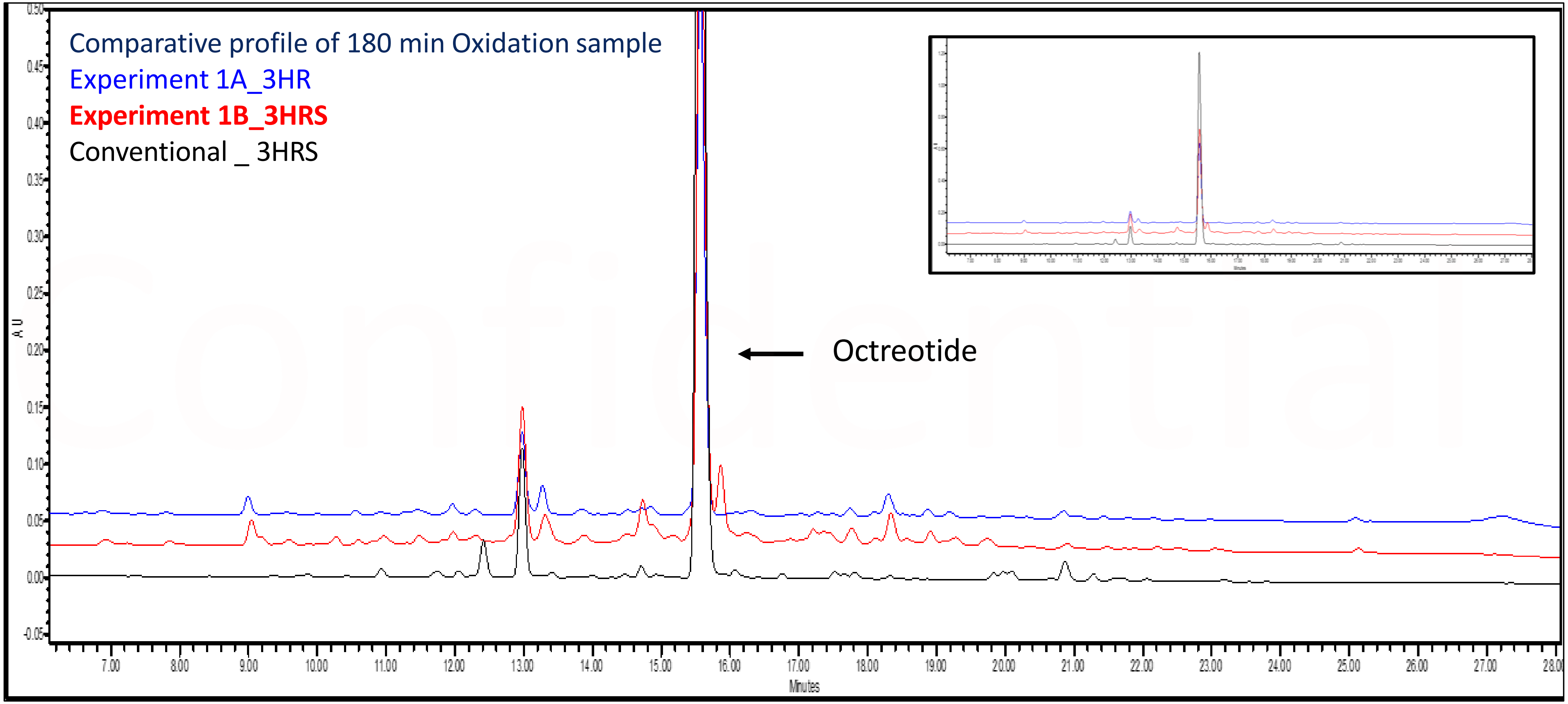
Greener approach in cleavage and oxidation process

Consumption of linear peptide to form cyclic		
Time point	1 - A Conventional	1- B Green
Before Oxi.	51.06	57.03
5 min	21.51	30.32
60 min	9.37	17.82
120 min	5.81	4.85
180 min	0.17	0.18



HPLC analysis of Crude Octreotide Samples



Process impact of Green-SPPS Vs. Conventional

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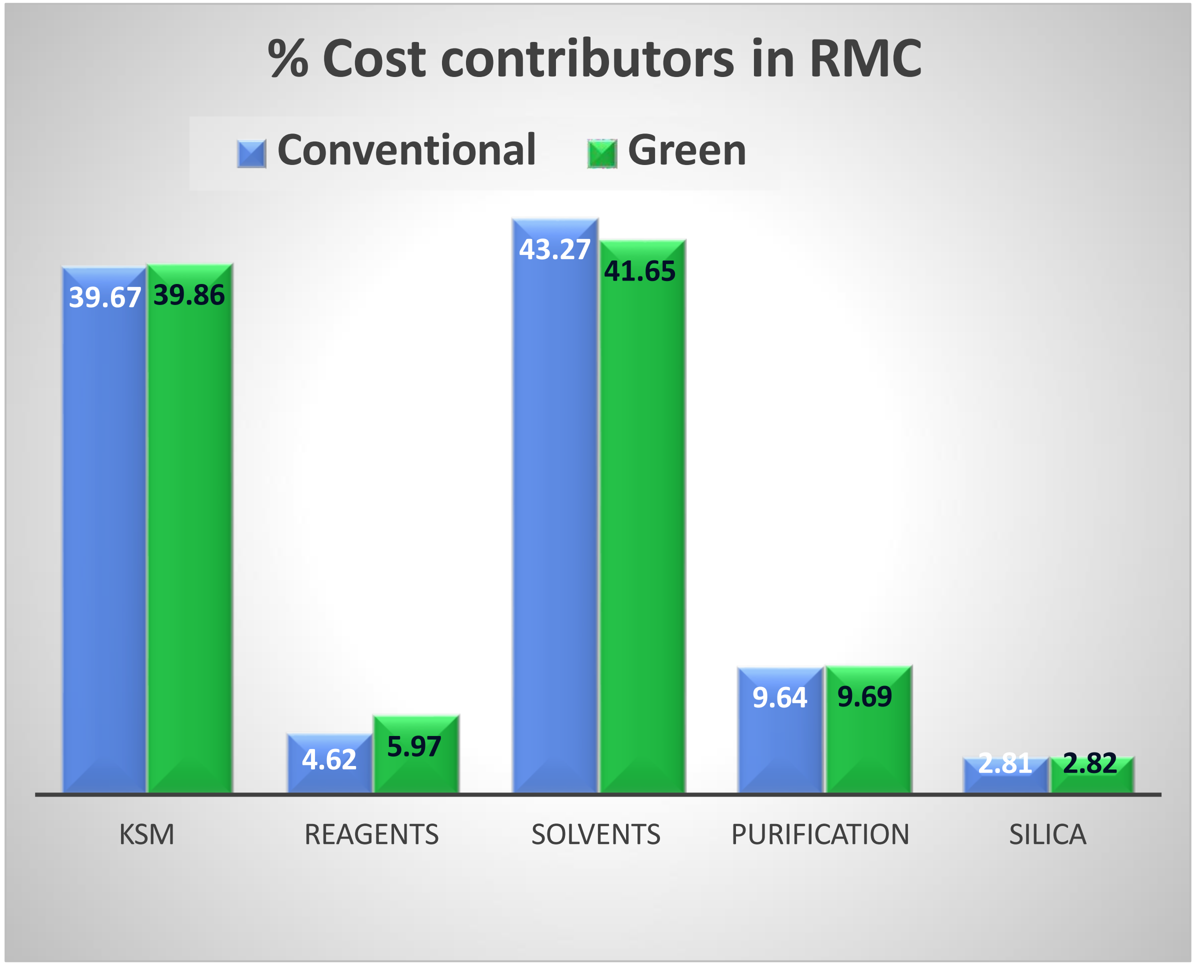
- Use of potent hazardous solvent DMF can be reduce to 60-80%
- Complete removal of DMF and DCM can not be possible for industrial approach
- Less hazardous coupling reagents T3P, Oxyma can give similar results but higher molar concentration required
- Peptides having lesser Amino acid (<10) shows identical results for both DMF and GVL solvents but impact on larger peptide sequence needs to be evaluate
- Particularly, in case of octreotide highly flammable ethers can be completely removed by quenching reaction mass in water. No major impact observed on quality

Commercial Aspects

% Cost contributors in Raw Material Consumption		
Raw Materials	**Conventional**	**Green**
KSM	39.67	39.86
Reagents	4.62	5.97
Solvents	43.27	41.65
Purification	9.64	9.69
Silica	2.81	2.82
Cost per Gram (USD)	Both has same RMC	

- **Lack of commercial availability of green Solvents create impact on Raw material costing on industrial scale.**



Use of non conventional technologies

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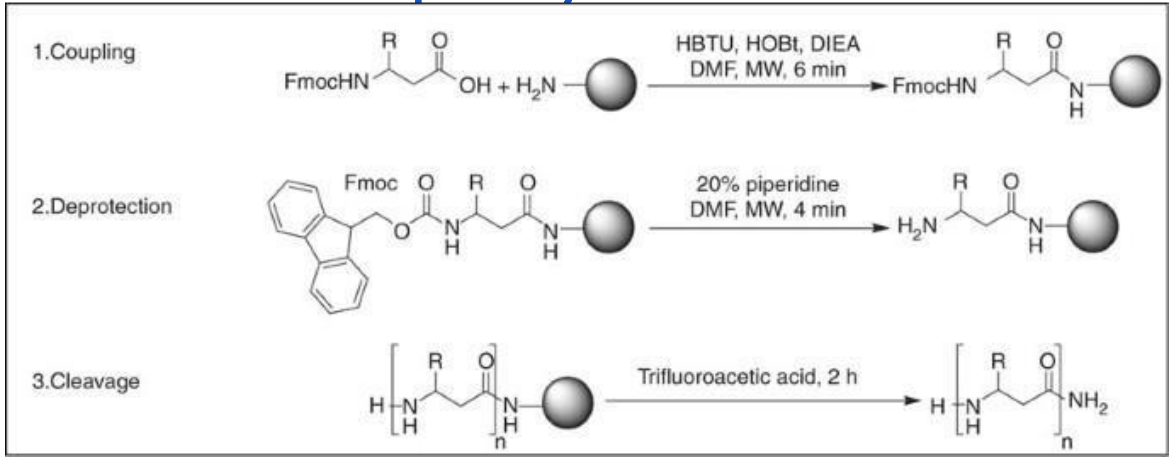
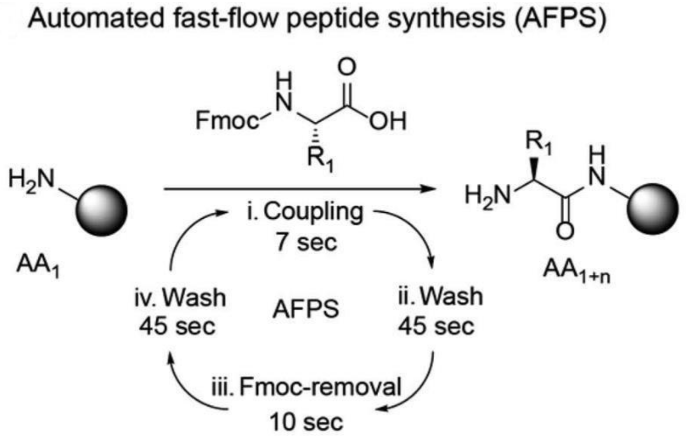
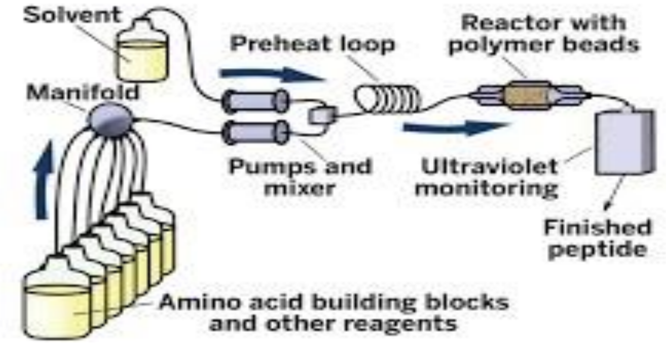
- **Microwave Peptide synthesis**



- **Automated fast flow Peptide synthesis**





Water soluble reagents for SPPS

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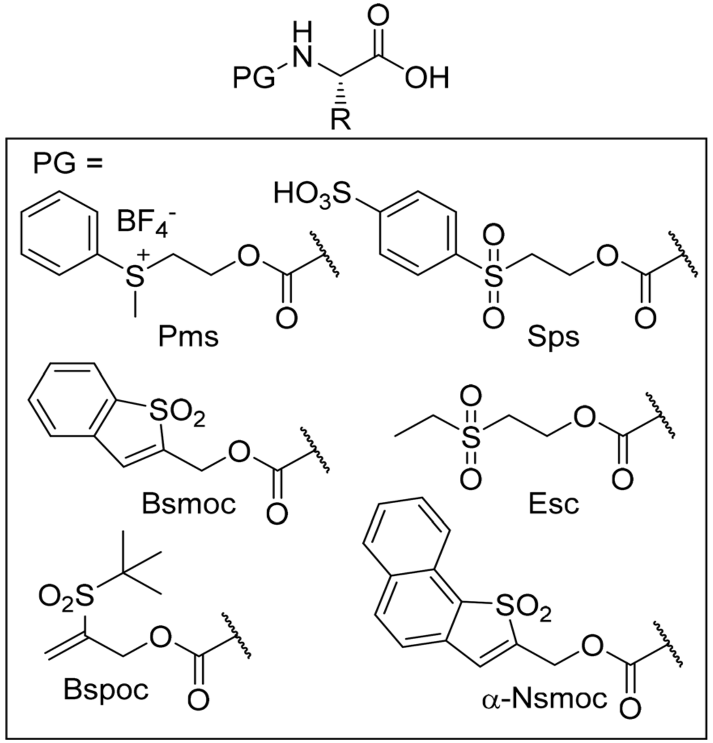
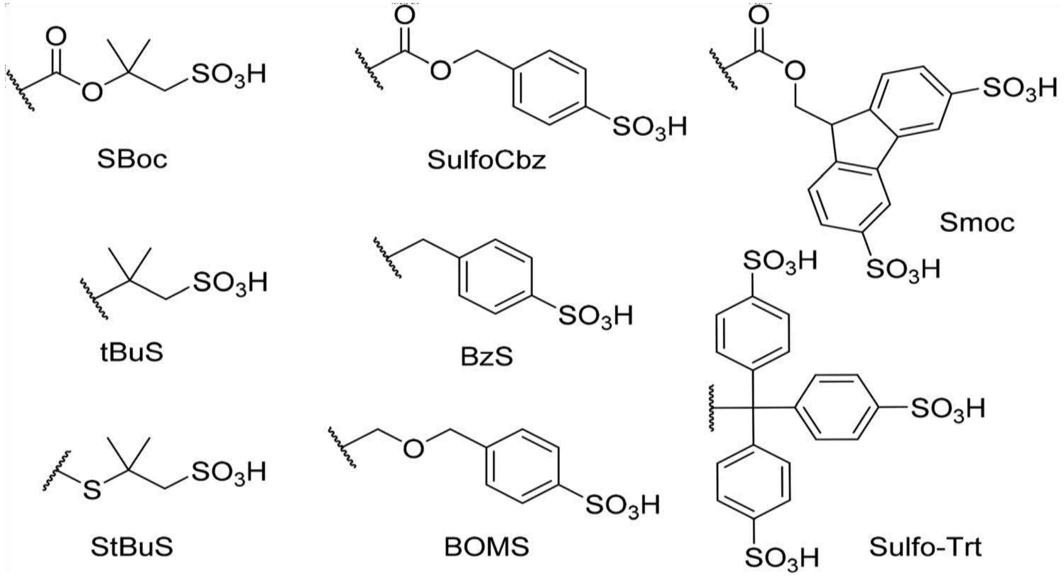
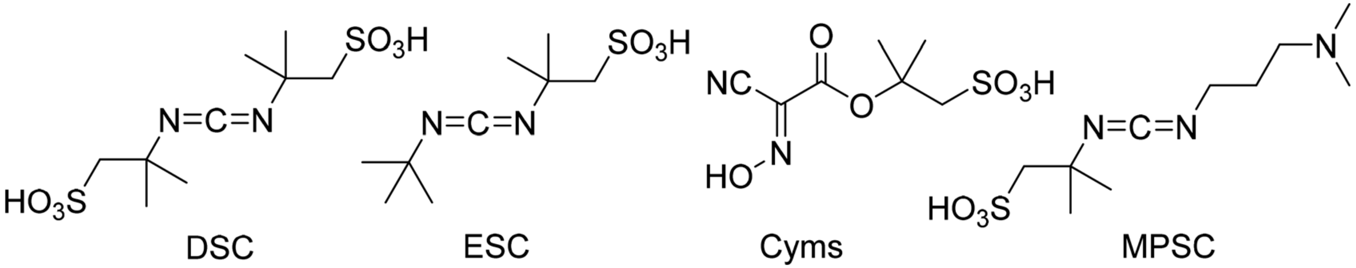
Water soluble Sulphonated protecting Groups





Water soluble coupling reagents



Points of consideration: Industrial use of Green SPPS

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- Changing the solvent system, ROS, use of new technology likely affect the purity profile of API
- New impurities generated in process will have to be qualify in new toxicology studies
- Changes are only made if there is a substantial financial incentive or if required by the authorities

Who we are

Enzene is an innovation-driven, technology-led differentiated biotech company offering integrated CDMO services for Biopharma

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Enzene Inc, New Jersey

Enzene Mammalian Mfg., Pune

Enzene Microbial Mfg., Pune

- **Enzene**, a subsidiary of **Alkem Laboratories Ltd.** and **VC-backed firm**, offers **fully integrated platform** from **Discovery and Development to Fill & Finish** across wide range of modalities
- We operate state-of-the-art **R&D facility** with Ambr 250 bioreactor and 8 more bioreactors[2] (2L-10L) and **cGMP manufacturing facilities** with 5 suites (20L-2000L) across fed-batch, semi-continuous & patented **continuous manufacturing, EnzeneX™ (among first movers globally)** We have **GMP facility** with supporting lab coming up in **US** (54,000 sq. ft.) by Q3 2024. We also have **discovery service** unit coming up in India by mid-2024
- Our **technical expertise, flexibility and tailor-made solutions,** regardless of project scope or scale, **makes outsourcing easy**
- **6 CLD (India), 1 CLD (Global), 2 PD (Global), 2 Pre-clinical (Global), 1 Pre-clinical (India), 6 Phase 1 (Global), 2 Phase 1 (India), 3 Phase 3 (India), 1 Phase 3 (Global), 7 Commercial (India)**
- **2 additional bioreactor orders have been placed; Delivery expected by Apr'23**

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Green chemistry is replacing our industrial chemistry with nature's recipe book. It's not easy, because life uses only a subset of the elements in the periodic table. And we use all of them, even the toxic ones.

Janine Benyus