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Барнаул (3852)73-04-60
Белгород (4722)40-23-64
Благовещенск (4162)22-76-07
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INNOVATIONS FOR TIDES

Powders, Processes and Progress

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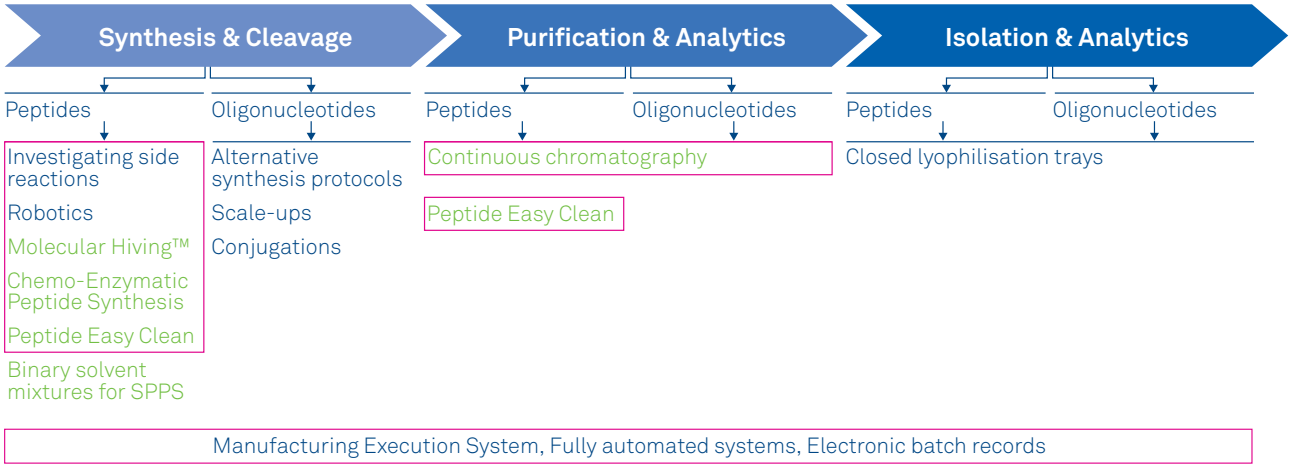
LIST OF ABBREVIATIONS

AEX	Anion Exchange Chromatography
API	Active Pharmaceutical Ingredient (drug substance in medicines)
Boc	t-Butoxycarbonyl (temporary amino-protecting group used for peptide synthesis)
CDMO	Contract Development and Manufacturing Organization
CEPS	Chemo-Enzymatic Peptide Synthesis
CMR	Carcinogenic, Mutagenic, or toxic for Reproduction
DTT	Dithiothreitol
DMF	Dimethyl formamide (solvent used in SPPS)
DMSO	Dimethyl sulfoxide
Fmoc	9-Flourenylmethoxycarbonyl (temporary amino-protecting group used for peptide synthesis)
GMP*	Good Manufacturing Practice
cGMP	current Good Manufacturing Practice
HFIP	Hexafluoro-2-propanol
HPLC	High Performance Liquid Chromatography (method of analysis and purification)
LPPS	Liquid-phase Peptide Synthesis
MES	Manufacturing Execution System
MCSGP	Multicolumn Counter-current Solvent Gradient Purification
MS/MS	Tandem Mass Spectrometry
NCE	New Chemical Entities (drug substance not yet licensed)
NMP	N-methyl-2-pyrollidone (solvent used in SPPS)
PEC	Peptide Easy Clean
PEG	Polyethylene glycol
SPPS	Solid-Phase Peptide Synthesis
TFA	Trifluoroacetic acid

* GMP = Good Manufacturing Practice, production guidelines that aid quality assurance. These guidelines must be followed when manufacturing substances to be used in humans. Adherence is regularly checked by specialist authorities (such as Swiss- medic in Switzerland or the FDA in the USA). Drug substances in medicines, ingredients in cosmetics and additives in foods are some of the items that are sold under these guidelines.

WE INNOVATE TIDES MANUFACTURING

Peptides and oligonucleotides (TIDES) have the potential to expand the space of "druggable" targets. The growing number of TIDES – under investigation in clinical trials and used as therapeutics – is accompanied by an increasing need for efficient and cost-effective routes for their large-scale manufacture. However, large-scale manufacturing of these substances involves many challenges from decreasing yields for longer peptides to the enormous amounts of solvents needed for purification. The only way to make the chemical production of these therapeutic powders efficient and sustainable is to improve the manufacturing processes. This can be achieved either through process optimizations or by new innovative technologies. In this brochure we highlight a few of our innovations as shown in the figure above.



As a CDMO Bachem has implemented a new level of automation and digitalization of solid-phase peptide synthesis (SPPS) to meet the predicted rise in demand of capacity and compliance. We have boosted our processes efficiency in terms of time, cost, safety, and reliability as you can read on the next page.

Shorter peptides can be produced without hazardous solvents, with more efficient scale-up and enhanced process controls using Molecular Hiving™ technology. To use this technology, we signed a licensing agreement with our partner Jitsubo CO., LTD. Under this agreement, Jitsubo's experts develop selected manufacturing processes using their Molecular Hiving™ technology and transfer these processes to Bachem. Further optimizations, scale-up, and commercial production are done at Bachem. The details of this technology are described on page 8.

In the production of larger peptides with more than 40 amino acids or even the production of small proteins, peptiligase enzymes offer a scalable alternative for large-scale manufacturing. We are investigating this together with our partner EnzyTag. As described on page 10, peptiligases enable a more economical manufacturing not only of large linear peptides, but also of cyclic peptide drugs and (bio)conjugates like peptide-oligonucleotide conjugates.

Purification can be a bottleneck in the production of oligonucleotides and peptides, because of the enormous amounts of solvents involved. We addressed these challenges with different approaches. We set up the first continuous chromatography system for center cut purification of peptides and oligonucleotides at industrial scale, as you can see on page 6. Together with our industry partner Belyntic we use Peptide Easy Clean technology to produce and purify peptides in a more sustainable and greener way as you may check out from page 12 on. Finally, we have investigated the side reactions that can occur during the manufacture of TIDES, as well as during their storage leading to loss of product. An example of our investigations can be found on page 14.

Our technological leadership and innovative strength have been the cornerstones of our success since the very beginning of our company more than 50 years ago. We are innovating TIDES manufacturing as we must! We hope this brochure can show you how your project could benefit from the innovative processes we are developing with our partners.

Examples of innovative technologies implemented at each step of peptide or oligonucleotide API manufacturing at Bachem. In green color: Our green chemistry success stories. The examples encircled in magenta are explained in more detail on the following pages.

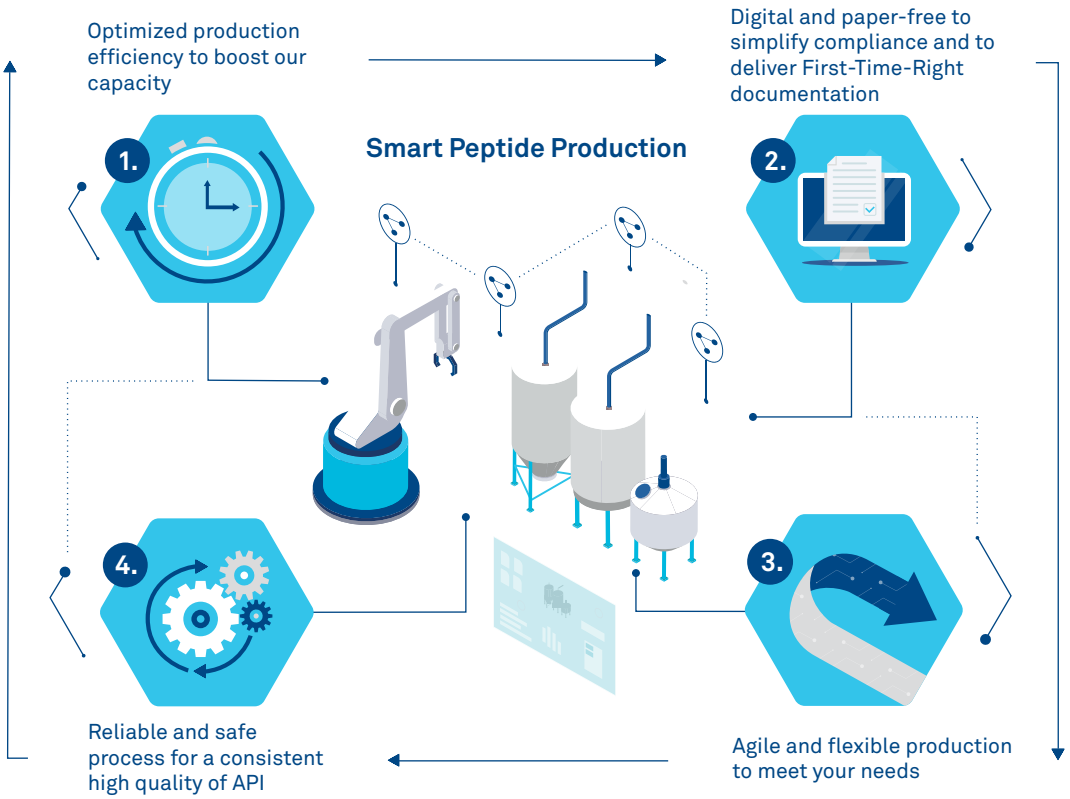
TOWARDS INDUSTRY 4.0 THROUGH THE AUTOMATION AND DIGITALIZATION OF SPPS

Industry 4.0 refers to the automation and digitalization of traditional industrial processes through smart technology and integration. An "Industry 4.0-smart factory" is characterized by machines that are interconnected, interoperable, and can process data autonomously. More automation leads to shorter production cycles, more consistent product quality, higher efficacy, and more precise documentation.

Bachem, known for establishing industry standards in innovative and efficient large-scale manufacturing processes for active pharmaceutical ingredients (API), is constantly improving its degree of automation. For example, our automated SPPS process allows operations to be carried out with minimal human intervention. This improved the reliability of the process, the reproducibility of its results and its safety, while significantly increasing its cost-effectiveness. We have also invested in digitalization of the production process. As results documentation can be conducted in a paper-free way, improved first-time right rates can be attained, and by – implementing a plant information system – data analytics is possible.

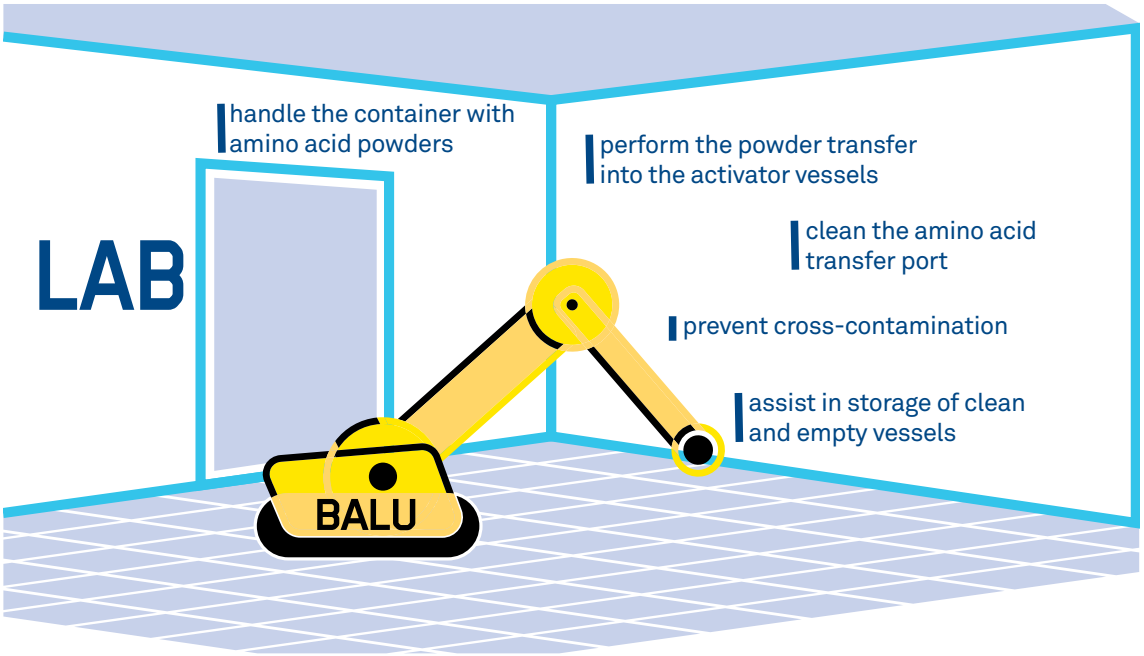
How it works

Our first robotic operator, the Bachem amino acid loading unit or BALU, was designed and programmed for mid commercial scale GMP SPPS (150 L vessel size). BALU performs all the tasks shown in the figure on page 5 without the involvement of a human operator and thus 24 hours, 7 days a week independent from shift systems. A barcode scanner that reads the labels placed on the amino acid containers ensures correct handling. The process control system of the factory floor with the robot is integrated with a Manufacturing Execution System (MES), that shares production data seamlessly across Bachem. Fast and efficient responses to any variations in the production process are therefore possible.



Advantages of our smart production of peptides via the automation and digitalization of SPPS at Bachem.

Another innovative feature of our fully automated SPPS process is Process Analytical Technology (PAT), which performs inline analytics after key steps. PAT removes the need for manual in-process controls, thus decreasing the need for human input, and provides better control of critical process parameters. This increases process safety, reproducibility, and



A first-of-its-kind robot for peptide manufacturing at Bachem.

accuracy. Implementing PAT for our process control decreases the need for human input and the cost of goods and leads to higher reproducibility in preventing side reactions.

The digitalization of our SPPS process started by integrating the process control system into the MES. The MES governs the process control system by defining the sequence of operations that must be performed, as written in a MES recipe, also called the Master Batch Record. Furthermore, the MES records all events, process values, alarms, as they happen during the process. Finally, the MES generates an electronic batch report. This means all equipment is managed without the need for physical logbooks. MES documentation is completely paperless while remaining fully GMP compliant!

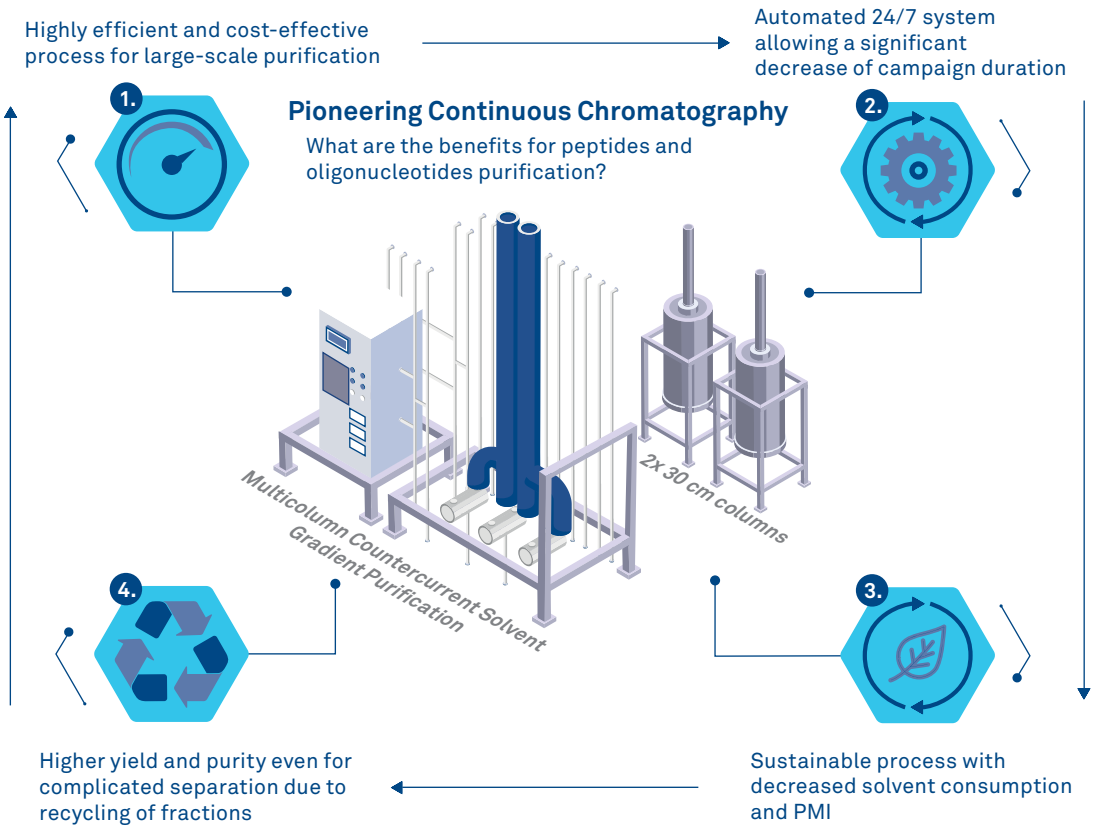
Full digitalization was achieved by connecting the MES to our Enterprise Resource Planning system. Execution of process orders, automatic take-out and stock creation, material flow, and inventory control are thus also fully automated and paperless. Finally, our digitalization strategy includes real- time logging and long-term archiving of data on a Historian platform. This plant information system allows for real-time and remote monitoring of the factory floor and easy access to data for trend and batch-to-batch comparisons. In addition, it provides the basis for future predictive and prescriptive data analytics.

At Bachem, further digitalization and automation initiatives are on the horizon. Through these innovations, we will maintain our technological leadership and set a high industry standard for customers.

Benefits

- Automation and digitalization are increasing our capacities with more flexible, reliable, and scalable manufacturing processes delivering consistently high-quality API.
- Thanks to our full automation of the SPPS process we can optimize equipment usage and reduce operating times.
- Full digitalization of the SPPS process allows for paperless documentation, improved first-time-right rates, data analytics and improved data safety and integrity.
- Our GMP documentation has been simplified and improved.

MAXIMIZING YIELD AND MINIMIZING WASTE: CONTINUOUS CHROMATOGRAPHY



Continuous chromatography: a game-changer for large-scale production of peptides and oligonucleotides.

Purification is not only paramount for achieving high purity but also a major determinant of the productivity of the entire API manufacturing process. The innovative Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) technology represents great progress in the downstream process for peptide and oligonucleotide manufacturing. Compared to single-column batch purification, solvent consumption is typically decreased by over 30%, thus contributing to a higher level of sustainability. The process has a higher capacity and often reaches the target product purity often with a higher yield, typically 10% more. The automated system runs 24/7 and has the potential for additional reductions in cycle time. In 2021, Bachem acquired the first two process scale MCSGP systems for the High-Performance Liquid Chromatography (HPLC) and Anion Exchange Chromatography (AEX) purification of peptides and oligonucleotides using 20 and 30 cm diameter columns. These systems are qualified for GMP use, and first purifications at scale have been successfully performed.

How it works

MCSGP is a fully automated system using two columns that are operated in countercurrent mode.

It internally re-purifies side-fractions, called side-cuts, while eluting product fractions by changing from one column to the other. During gradient purification, weak and strong adsorbing impurities are separated from the product.

We have proved that MCSGP technology is cost-effective and reduces waste. Sharing the benefits of continuous chromatography with the pharma or biotech companies that rely on us for their products' excellence is part of our mission. MCSGP is a greener, economical, and more sustainable process.

Benefits

- MCSGP increases the capacity, quality, and sustainability of the purification process.
- It is a scalable, highly efficient technology, that is particularly useful for large-scale production.
- Using an automated system, it can run 24/7, allowing a significant decrease in purification campaign cycle times.
- More sustainable purification is possible with MCSGP, as it reduces solvent consumption and process mass intensity.
- It deploys standard chromatographic conditions, and API quality is not adversely affected by changing from batch mode to continuous mode.

Continuous chromatography equipment for API purification at the Bachem site in Bubendorf.

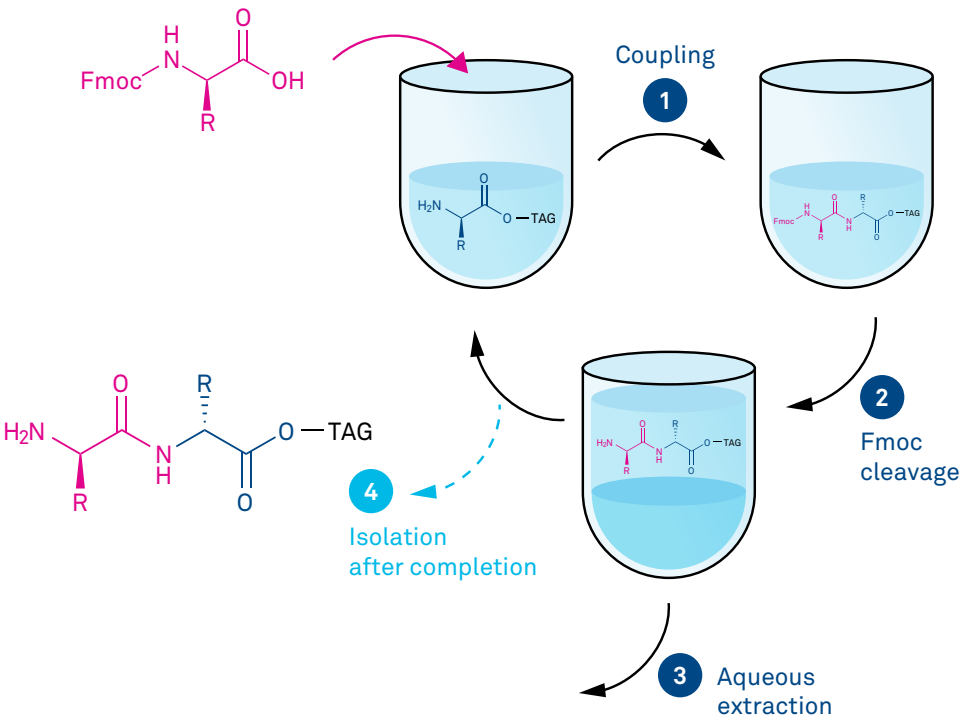
MAKING PEPTIDES WITHOUT HAZARDOUS SOLVENTS: MOLECULAR HIVING™

We aim to redesign our processes to minimize the use or generation of hazardous substances and their environmental impact. For this reason, we have implemented innovative solutions for greener peptide synthesis by replacing potentially harmful solvents whenever possible. Jitsubo's Molecular Hiving™ technology is one example of that approach. This technique is most advantageous for shorter peptides with up to 10 to 15 amino acids, that are required in larger quantities with batch sizes of 5 to 10 kg.

With Molecular Hiving™, fewer washing and filtration steps are needed compared to classic peptide synthesis, resulting in a reduction up to 60% in the use of organic solvents. Additionally, solvents and reagents classified as carcinogenic, mutagenic, or toxic for reproduction (CMR substances) can be entirely avoided. This makes the peptide products manufactured using this technology highly attractive for the pharmaceutical and cosmetics industries. Molecular Hiving™ can be applied for both GMP and non-GMP products and processes.

How it works

Molecular Hiving™ is a method of liquid-phase peptide synthesis using a hydrophobic anchor, onto which the peptide is assembled in the same way as on the resin in SPPS. This anchor, also called a tag, is soluble in organic solvents but insoluble in aqueous solutions. This is especially important, as all applied reagents, excess amino acid derivatives, and other byproducts are removed by aqueous extractions during synthesis. The anchor-bound peptide always remains in the organic phase, and only at the very end, when it has been fully assembled, are the peptide and its anchor isolated from the solution through a precipitation step. Cleavage of the peptide from the anchor and of the protecting groups from the peptide are performed in an analogous way to SPPS, using a trifluoroacetic acid (TFA) cleavage cocktail. The solubility of the peptide on the tag defines the length of the synthesized peptides, as issues regarding solubility in non-water miscible solvents are often observed for longer peptides and the peptide must remain soluble in the organic phase throughout the entire process. However, longer peptides can be made using a fragment approach.



Important steps of the Molecular Hiving™ technology.

Typical equipment used for Molecular Hiving™ at the Bachem site in Bubendorf.

The Fmoc amino acid derivatives are coupled by adding them directly to the solution (step 1). Thus, direct monitoring of the reaction's progress is possible using HPLC. There is always a certain amount of water in the organic phase, and because of that, this technology is not suitable for water-sensitive coupling reagents. However, there is a wide range of coupling reagents available that can be used for Molecular Hiving™. Typically, fewer equivalents of Fmoc amino acid derivatives are needed than in normal SPPS, as the reaction in solution has a faster conversion rate.

When the coupling is complete, Fmoc cleavage (step 2) is performed by adding a suitable base. Again, monitoring by HPLC is possible, so it is easy to directly monitor control whether all Fmoc protecting groups are cleaved. After this, aqueous extractions (step 3) will remove the excess amino acids, coupling reagents, and bases.

Steps 1 to 3 are repeated depending on the length of the desired peptide, each cycle adding one amino acid to the chain. After the peptide is fully assembled, the protected peptide on the tag is isolated by means of a precipitation process (step 4). Finally, the peptide is cleaved from the tag, along with its protecting groups, and purified if necessary.

Benefits

- This is a CMR free process by which peptides can be synthesized without the use of DMF, NMP, or any other hazardous solvents and reagents.
- There is a significant reduction in solvent consumption compared to SPPS processes, and to traditional peptide synthesis in solution (LPPS).
- Compared to SPPS, fewer equivalents of Fmoc amino acid derivatives and coupling reagents are required.
- Direct in-process control is possible, e.g., by HPLC.

WHEN ENZYMES DO A BETTER JOB: CHEMO-ENZYMATIC PEPTIDE SYNTHESIS

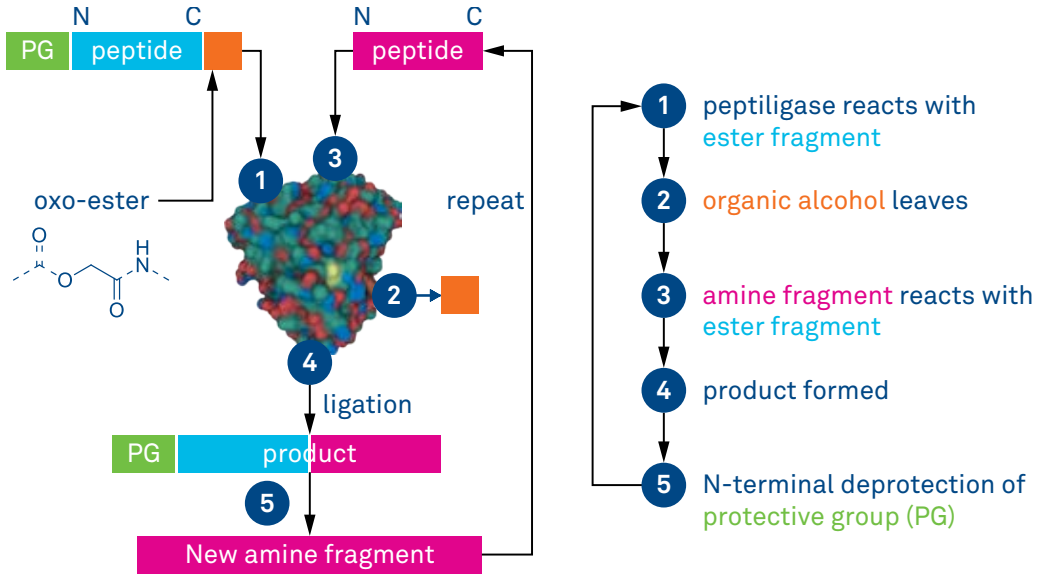
Therapeutic peptides are becoming ever longer and more complex. To make these peptides in large quantities we are working with our industry partner EnzyTag to develop an efficient enzymatic ligation tool called chemo-enzymatic peptide synthesis, or CEPS. It is a state-of-the-art, greener, and more sustainable synthetic way to make cyclic or long peptides or even small proteins that are difficult to express recombinantly. For that purpose, CEPS uses a peptide ligating enzyme, also known as peptiligase, to form longer peptides by coupling shorter peptide chains previously produced by SPPS.

Fmoc-based SPPS routinely delivers peptides with a length of 50 to 60 amino acids. Longer sequences are difficult to make because of the impurity levels. CEPS enables the synthesis of peptide sequences well above 100 amino acids. Due to the fragment approach the impurity profile is excellent. The process runs under aqueous conditions at near neutral pH as the enzyme is active in the pH range of 7 to 8.5. The enzyme is derived from an endotoxin-free biotechnological process in Bacillus subtilis bacteria, which are generally regarded as safe (GRAS) organisms.

How it works

Peptiligase originates from a serine protease – an enzyme that normally cleaves peptide bonds. The enzyme has been engineered in such a way that it now creates peptide bonds. This was achieved by substituting the active site serine with a cysteine. Peptiligase was also designed to recognize multiple sequences of amino acids, making CEPS a traceless technology without the need to insert a specific recognition sequence. Furthermore, the enzyme shows good activity and stability in the presence of organic co-solvents or denaturing agents, which enables the efficient ligation of hydrophobic and/or folded peptides.

To make a peptide by CEPS, at least two peptide fragments are used. The first fragment is a synthetic peptide that needs to have an oxo- or thioester at its C-terminus. The ligation starts with the reaction between the ester and the thiol of the active site cysteine (step 1 in figure above). As a result, organic alcohol leaves the active site (step 2), and the peptide is covalently attached to the peptiligase via a thioester. The amine fragment (synthetic or recombinant) then comes in and replaces the thioester, forming the product (steps 3 and 4). The peptiligase is then free



Ester fragment requires **4 amino acids** for proper docking
Amine fragment requires **2 amino acids** for proper docking
(most amino acids are tolerated except proline)

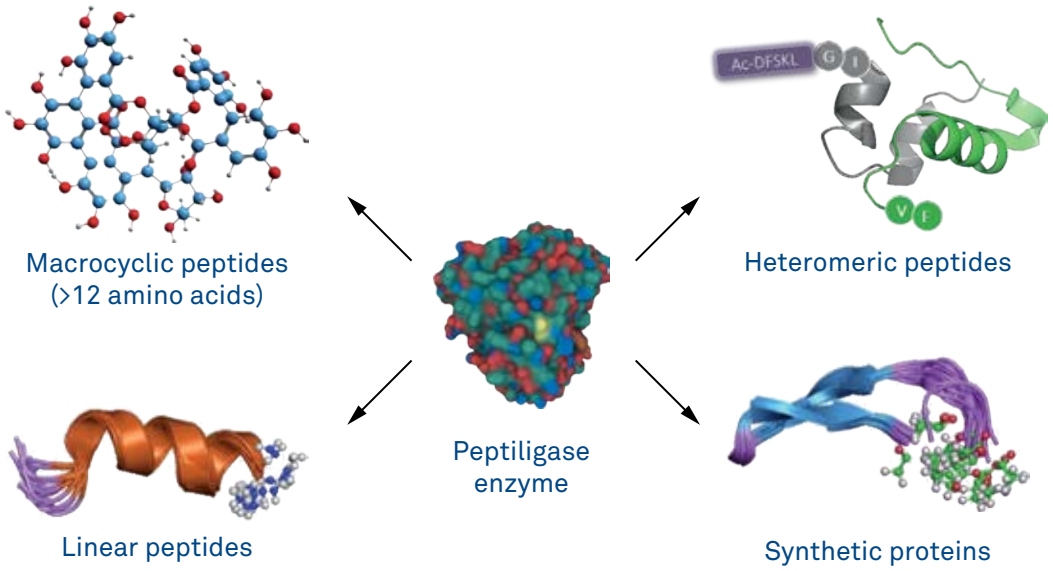
The CEPS process.

to catalyze the next cycle of conversions. The product can subsequently undergo a second ligation cycle with a different ester fragment. Here, the product serves as a new amine fragment, provided that the "new" N-terminus is first deprotected.

Peptiligase requires six amino acids for proper docking: four amino acids in the ester fragment and two in the amine fragment. The full range of canonical amino acids, including hydrophobic amino acids, is tolerated in the active site. Outside of the active site, non-canonical amino acids and non-peptide motifs can also be included in the peptide sequence. To produce cyclic peptides and cyclotides, peptiligase needs at least twelve amino acids for cyclization, given that six amino acids are needed for recognition in the reaction pocket of the enzyme and another six are needed to close the loop.

Benefits

- CEPS enables the regio- and stereoselective synthesis of peptides that cannot be (efficiently) manufactured by stepwise SPPS.
- In combination with SPPS long peptides with more than 40 amino acids and cyclic peptides with more than twelve amino acids can be made in high purity.
- Protection of side-chain functionalities is not necessary, as side reactions and racemization are absent.
- CEPS is more environmentally friendly, as the consumption of organic solvents is reduced.
- This is a scalable process applicable for GMP manufacturing.
- It is capable of synthesizing > 90% of marketed pharmaceutical peptides.



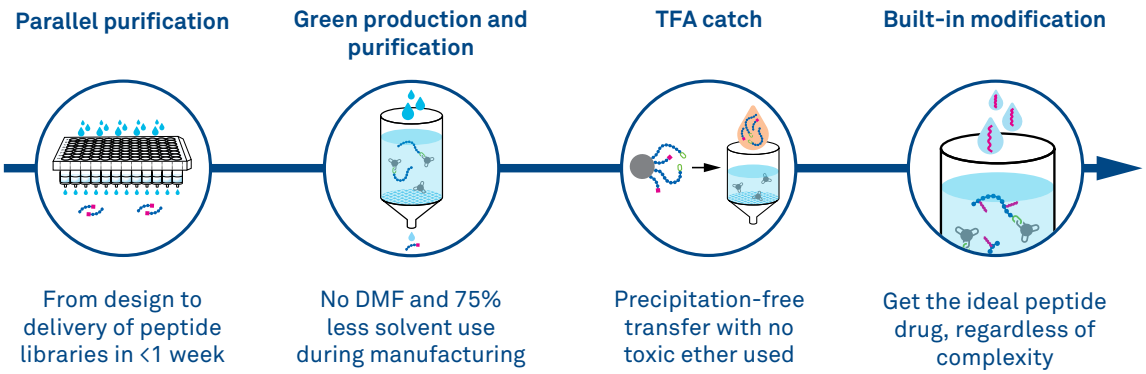
Types of peptide-based therapeutics that can be made by CEPS.

CATCH-AND-RELEASE SYNTHESIS AND PURIFICATION: PEPTIDE EASY CLEAN

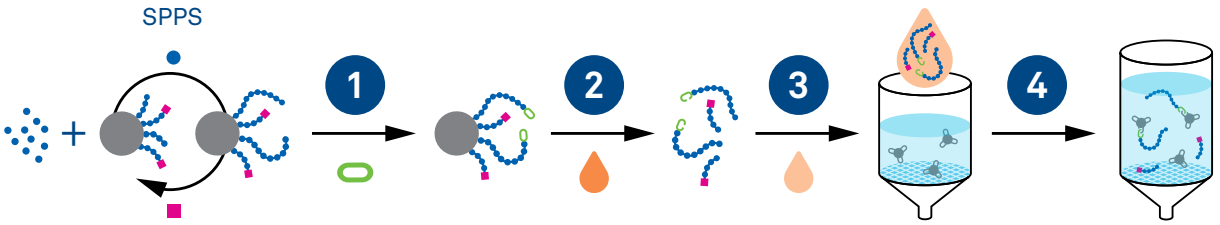
In collaboration with our industry partner Belyntic, we are addressing the bottleneck of sequential peptide purification by RP-HPLC. Belyntic's Peptide Easy Clean (PEC) is a catch-and-release platform, that enables the purification of even the most challenging peptides, e.g., long, hydrophobic, and aggregating peptides, in parallel. PEC not only speeds up purification, but also reduces solvent consumption and enables further chemical modifications of peptides.

How it works

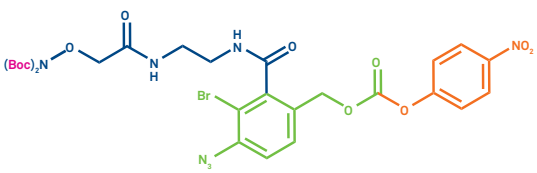
The core of the innovation is a new traceless linker for catch-and-release purification that contains a leaving group for binding to the peptide, a cleavable unit, and a protected catch tag (see figure above). This linker is the first of its kind, providing a catch-and-release purification method that is applicable across all peptide sequences. The non-hazardous, stable powder is available in large scale for API production.



The advantages of Belyntic's Peptide Easy Clean within the peptide manufacturing process.



When using PEC for purification and/or modification of peptides, the acetic anhydride capping steps during the preceding SPPS are key factors. Capping is required to deactivate any unreacted site on the growing chain of the peptide. Coupling of the reductively cleavable PEC purification linker is straightforward and can be automated using a peptide synthesizer (step 1). Next, the peptide is cleaved from the SPPS resin using a TFA cocktail (step 2).



Structure of the catch-and-release linker for peptide purification and modification with the Boc-protected oxyamine (catch-tag) for immobilization (pink), an aryl-azide-core for safety-release (green) and an activated carboxylic acid ester for coupling to the N-terminus of the peptide (orange).

This also activates the PEC-linker for the catch step. Scavengers are used to prevent the re-addition of protecting groups on the target peptide.

PEC allows the use of solvents such as dimethyl sulfoxide (DMSO) or hexafluoro-2-propanol (HFIP) and chaotropic additives for dissolution (step 3). Moreover, TFA mixtures or even the TFA cleavage cocktail itself can be directly applied within PEC purification processes. This can significantly improve handling of the crude peptide by preventing self-assembly and the formation of aggregates.

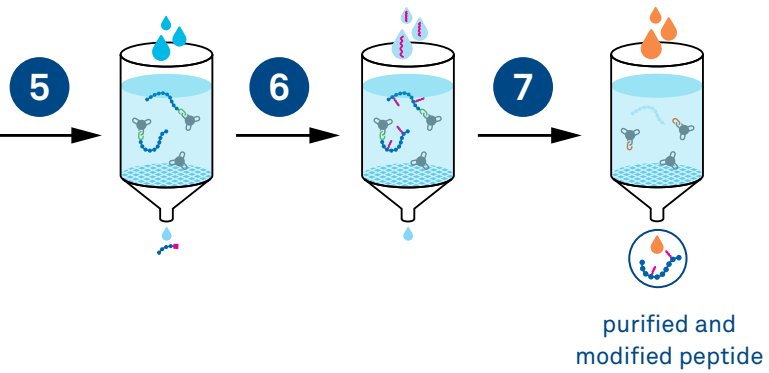
For the catch step (4), the linker-modified target peptide is covalently attached to agarose beads via rapid and robust oxime ligation

Then, through repeated washing steps (step 5), all unmodified and truncated sequences, and chemical impurities, are removed. At this stage, the unprotected peptide bound to the purification platform is ready to undergo solid-phase modifications, such as lipidation, disulfide formation, thioether stapling and other click-type reactions. This is circumventing the difficulties with modification that are encountered in the solution phase and subsequent HPLC purification (step 6).

The last step (7) is the traceless safety-release of the purified full-length peptide from the linker via reduction with dithiothreitol (DTT) solution, followed by DTT (ox) washout and weak acid-induced elimination. Finally, the pure peptide is recovered after washing steps and optional ether precipitation.

Benefits

- Peptide Easy Clean is universally applicable, enabling the efficient manufacturing of new peptide-based drug modalities.
- It allows parallel purifications, in contrast to RP-HPLC which is a sequential process.
- Large-scale API production with Bachem as partner is possible.



The PEC technology with the steps: PEC-linker coupling (1), TFA cleavage and precipitation (2), dissolution (3), catch = covalent capture (4), washing (5), optional modification (6) and reductive safety-release (7).

INVESTIGATING IMPURITY FORMATION

One of the most widely used strategies for the covalent modification of peptides and proteins is the thiol-maleimide reaction. This reaction is used to add chemical labels onto biomolecules via thiol conjugation, such as fluorescent dyes, polyethylene glycol, radiolabels, and small molecules. The advantages of this reaction are the rapid reaction kinetic between maleimides and thiols and the preferential chemical reaction at a neutral pH. However, there are also disadvantages, like the various side reactions that occur (see figure below). One of these – underreported in the literature – can complicate the purification, characterization, and storage of peptide conjugates leading to loss of product and complex characterization: thiazine formation during the conjugation of N-terminal cysteine peptides to maleimides. Chemists at Bachem have studied this side reaction to establish conditions that can prevent thiazine formation, if required.

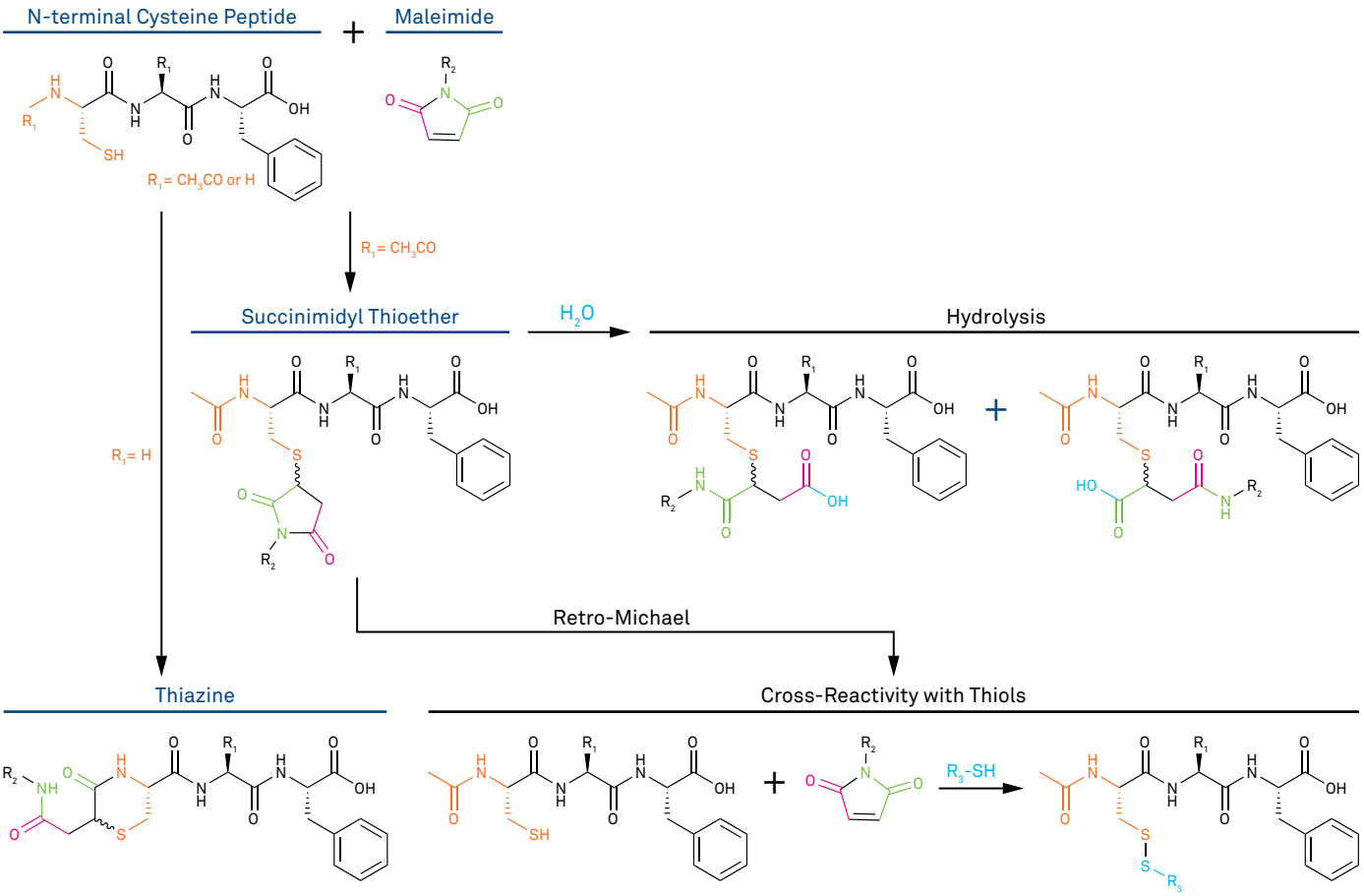
Thiol-maleimide coupling is well established but prone to various side reactions.

How it works

By investigating the pH dependence and sequence dependence of succinimidyl thioether using a tripeptide model system, a significant increase in the rate of thiazine formation was observed at basic pH. This was consistent with previous work indicating that the rearrangement is promoted under basic conditions. The considerable increase in the rate of thiazine formation at higher pH supports a base-dependent mechanism involving a nucleophilic attack on the succinimide by the N-terminal amine.

When the amino acid adjacent to the N-terminal cysteine was substituted by various amino acids, generation of the thiazine impurity was observed in all instances, although the rates of thiazine formation differed depending on the tripeptides being studied. Even a maleimide linker which we expected to be more stable, resulted in substantial thiazine formation. This was further evidence that the side reaction is general. The presence of thiazine has been confirmed using different analytical methods.

Protonation of the N-terminal amino group under acidic conditions prevents the nucleophilic reaction and therefore the formation of thiazine impurities.



We keep processes under control.

Although conjugation under mild acidic conditions near pH 5 prevents thiazine formation, the subsequent purification, storage, and application of the peptide conjugates must be performed under acidic conditions to avoid the loss of succinimidyl thioether. Alternatively, acetylation of the N-terminal cysteine can be performed to prevent the formation of the thiazine impurity. Regarding the general nature of the thiazine side reaction, if the thiazine rearrangement needs to be avoided, we recommend avoiding the use of N-terminal cysteine in peptide conjugates, when succinimide thioether linkage is desired.

Beneficial outcome

- Performing the thiol-maleimide conjugation reaction under mild acidic conditions or avoiding the use of an N-terminal cysteine with a free amino group prevents the formation of the thiazine impurity.
- Also purification, isolation, storage, and application of the peptide conjugates should be performed under acidic conditions.
- We have the expertise in peptide chemistry to propose the right chemical process to our customers, thus ensuring that their peptides can be smoothly produced and time losses due to chemical issues are avoided.

BACHEM

GLOBAL BUSINESS

Bachem facilities are located in Switzerland, the EU, and in the USA. All cGMP manufacturing sites are inspected by the US-FDA and national authorities.

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