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Very Important Publication

Merging Continuous Flow Technology, Photochemistry and Biocatalysis to Streamline Steroid Synthesis

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Abstract: Since their structural elucidation in 1935, the introduction and substitution of functional groups and the modification of the steroidal scaffolds have been a fertile ground of research for synthetic and medicinal chemists. The discovery of steroids with hormonal and pharmacological activity has stimulated tremendous efforts to the development of highly selective and efficient synthetic procedures. Despite the progress made, steroid chemistry remains challenging and the preparation of steroidal compounds of pharmaceutical interests and in clinical practice, often requires long and elaborated synthesis. In recent years, a new impetus in the field came with the advent of enabling chemical technologies, such as continuous flow chemistry, which are exploited to overcome problems that arise from batch synthesis. Although it is still a niche sector, the use of flow technology in steroid synthesis and functionalization holds the premise to empower methodology development and to provide innovative tactics also for many hitherto uncharted chemistries. In this review, scientific contributions are reported and discussed in terms of flow set-up and advantages offered concerning process efficiency, optimization, waste minimization, safety improvement, easy scale-up and costs. We also highlight the main challenges, key improvements and the future trajectory in the application of continuous flow chemistry and its implementation to different disciplines such as photochemistry and biocatalysis with the ultimate goal of streamlining steroid synthesis.

Keywords: Continuous flow chemistry; Biocatalysis; Design of experiments, Enabling chemical technologies; Photochemistry; Synthesis; Steroids

1. Introduction

Steroids comprise a large and diverse family of natural products with crucial functions in vivo, such as physiological regulators, hormones, and provitamins.[1] They include sterols, sex and adrenocorticoid hormones and cardiac glycosides. Structurally, steroids are characterized by the skeleton of the cyclopentanperhydrophenanthrene core with methyl groups normally present at C10 and C13 positions (Figure 1). An alkyl side-chain may also be present at C17 position as in cholestanes. Compounds obtained from one or more bond scissions, ring expansions or contractions are generally classified as steroid derivatives (e.g., vitamins D). Owing to their plethora of chemical structures and biological activities, steroids play a prominent role in medicine and drug discovery.[2] More than 300 steroid-based pharmaceuticals have been registered[3] and are used as drugs and several steroids are under development. Nowadays, the global market of steroidal

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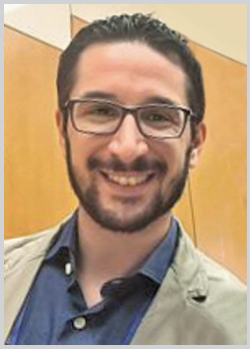
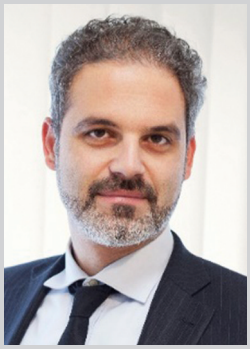
Francesco G. Mutti is professor of biocatalysis at the University of Amsterdam (Netherlands). He graduated in Industrial Chemistry (2004) at the University of Milan (Italy). After obtaining his PhD in Chemistry (2008), he was research associate in the groups of Prof. W. Kroutil at the University of Graz (2009–2012) and Prof. N. Turner at the University of Manchester (UK; 2013–2014). Since 2015, he is the group leader of the Biocatalysis at the University of Amsterdam. Among the others, he has been a Marie Skłodowska-Curie fellow and received an ERC Starting Grant. His main research interest is on the development of biocatalytic cascades for the sustainable synthesis of chemicals, enzyme discovery and engineering, continuous flow biocatalysis, biocatalysis in vivo and bio-electrochemistry.



Bruno Cerra graduated in Pharmaceutical Chemistry and Technology (2011) and obtained his PhD in Medicinal Chemistry (2015) at the University of Perugia. After some post-doctoral experiences, in 2021 he moved in Milan to the manufacturing company Steroid. Since 2022, he has a tenure track position at the University of Perugia. His research interests are focussed on steroid synthesis, process and flow chemistry.



Antimo Gioiello is professor of medicinal and synthetic organic chemistry at the University of Perugia (Italy). After graduating in organic synthesis (2001), in 2005 he obtained his PhD in medicinal chemistry at the University of Perugia. Antimo Gioiello leads several academic and industrial collaborations and his work experience spans various stages of drug discovery. He is also a co-founder of Tes Pharma, a research-based biopharmaceutical company focussed on delivering first in class preclinical candidates for novel therapeutic targets. His main research interests include steroid chemistry, lead discovery and development, new processing methods, and enabling chemical technologies such as continuous flow chemistry.

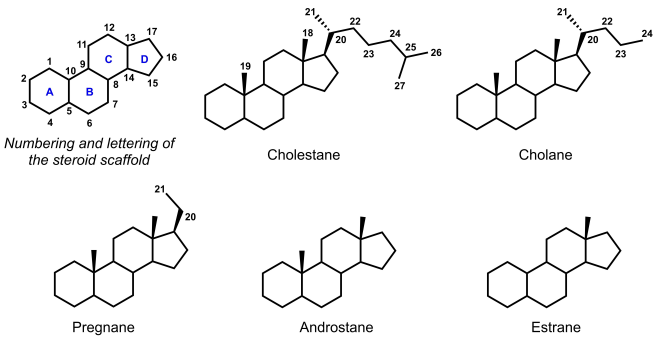


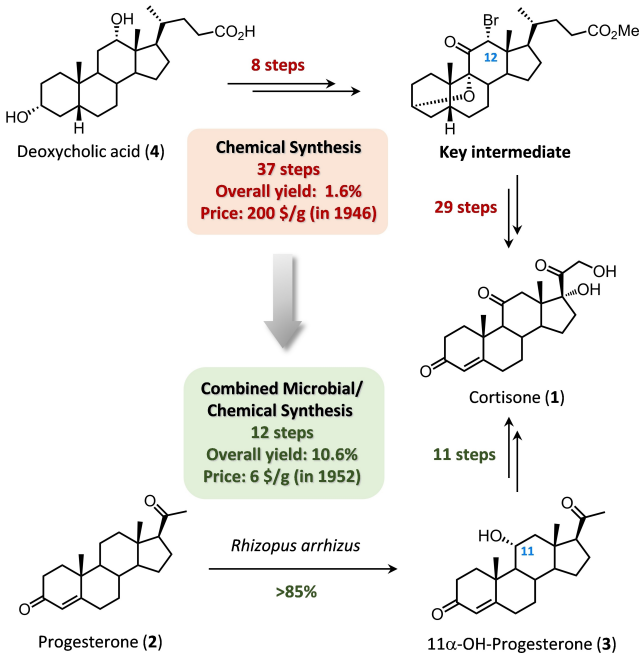
Figure 1. General structure, numbering and lettering of major steroids.

drugs and intermediates was estimated to be $4.85 billion in 2021[4] and it is expected to grow up to $7.66 billion in 2029.[5]

The breakthrough in steroid research came in 1949 with the discovery by Hench at the Mayo Clinic of the therapeutic efficacy of cortisone (**1**) for the treatment of rheumatoid arthritis.[6]

In the following decade, other steroids found clinical applications as diuretics, antibiotics and contraceptives opening up new therapeutic possibilities. During this period, also called the 'golden age of steroids', world's leading chemists, such as Robert B. Woodward, Derek H. R. Barton, Robert Robinson, Carl Djerassi, to name a few, were attracted to the field to make relevant steroids available for therapy and drug production.[7] As a result of these extraordinary worldwide efforts, highly selective and efficient synthetic procedures were developed for the chemical manipulation of complex polyfunctional steroids. It can be fairly stated that every novel synthetic method was tested and applied on steroids providing a better understanding of the mechanisms and reactivity, as well as milder and more selective reactions. Needless to say, steroid synthesis and manipulation require addressing many challenges that would have not been solved without innovations in synthetic methodologies

and technologies. For instance, the C11-oxygen that is distinctive of rare adrenal hormones and corticosteroids, including cortisone (**1**), is not found in any abundant natural steroids limiting the synthetic approaches to the use of precursors characterized by functional groups in or nearby the ring C. Initially, cortisone (**1**) was prepared from the bile acid 3α,12α-dihydroxy-5β-cholan-24-oic acid (deoxycholic acid, **4**) in 37 steps with low yield (0.16%) and high costs (200 $/g, 1946) (Scheme 1).[8] The search for alternative processing methods by competing pharmaceutical companies such as Merck, Upjohn, Squibb, and Pfizer led to the identification of more practical synthesis of 11-oxosteroids, some of which included the use of biocatalysis. For instance, in 1952, Peterson and Murray at Upjohn discovered that fermenting *Rhizopus fungus* ATCC 11145 was able to hydroxylate directly the C11 position of progesterone (**2**) markedly reducing the number of synthetic steps (Scheme 1).[9] By then, 11α-hydroxyprogesterone (**3**) became readily available at more than 30-fold lower production costs (6$/g, 1952) and on a large scale thereby solving most of the issues associated with the previous approaches and reshaping steroid research strategy throughout the pharmaceutical industry. The importance of the Murray-Peterson discovery was many-fold. Above all, it stimulated the discovery of a series of cortisone analogues of greater potency. Secondly, it evidenced the importance and the need of introducing innovation and technology to enable forbidden chemical transformations and obtain complex natural derivatives that are relevant for drug discovery and development.



Scheme 1. Comparison between chemical and biochemical processes of cortisone (**1**) from deoxycholic acid (**4**) and progesterone (**2**).

Due to the growing importance as pharmaceuticals, steroid chemistry represents a dynamic area of research both in academia and industry. The majority of steroidal active pharmaceutical ingredients (steroidal APIs) are currently produced by partial synthesis from naturally-occurring precursors often by multistep, time-consuming, and high waste-producing synthesis.[10] Therefore, steroid pharmaceutical industry urgently demands for novel strategies and improved protocols of well-established methods to meet modern synthesis criteria such as sustainability, safety, and economic profitability. In this review, we attempt to summarize some of the recent advances in the synthesis of steroids that have been enabled by continuous flow chemistry and its integration with other technological solutions such as catalysis, photochemistry, biotechnology and automation.

2. Concepts and Benefits of Continuous Flow Technology

In recent years, continuous flow chemistry has attracted a great deal of attention being a technology with a broad applicability to many different areas ranging from pharmaceutical and agrochemical, to petrochemical and fine chemical production.[11] Especially from a pharmaceutical perspective, continuous flow chemistry arguably represents one of the most innovative approach with the power to significantly improve chemical synthesis and to accelerate the journey from early drug discovery to manufacturing. Telescoping compound synthesis with biological evaluation using flow platforms provides opportunity to remove spatiotemporal boundaries, long feedback timing, and delay from compound design to data analysis. Moreover, flow chemistry offers fast and simple scale-up strategies. Indeed, the production capacity of a flow system can be raised by increasing the size of the reactor (scaling-up), the number of reactors running in parallel (numbering-up), and by running the reaction for longer (scaling-out). Therefore, the scale-up from gram to kilogram quantities of flow processes requires minimal chemistry modifications or reactor engineering.

In flow chemistry, a chemical reaction is performed using a network of interconnecting channels where they join one another, the fluids come into contact and the reaction takes place. A flow synthesizer consists of diverse interconnected and modular units adaptable with diverse combinations and set-ups by linking tubes to the respective flow zone.[12] A typical set-up includes diverse basic zones (Figure 2): solution and reagent delivery, mixing, reactor, work-up, monitoring/analysis, purification and collection.

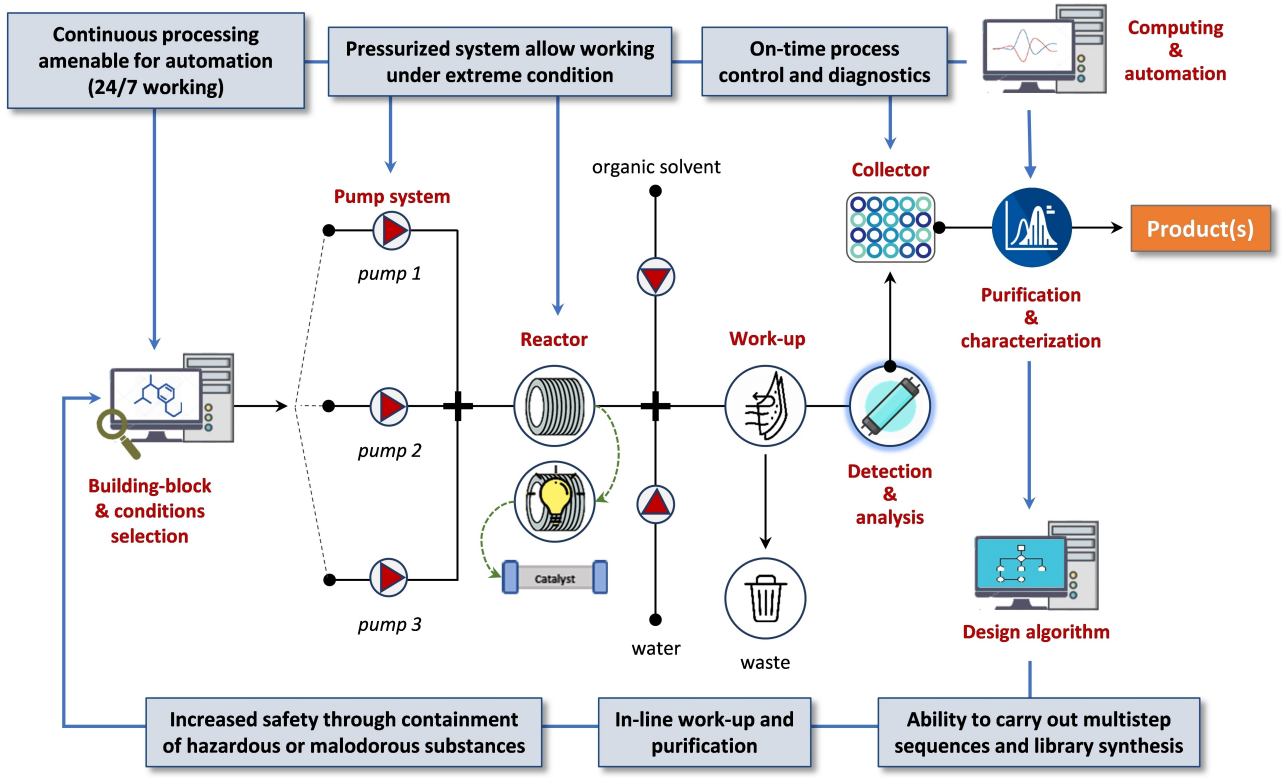


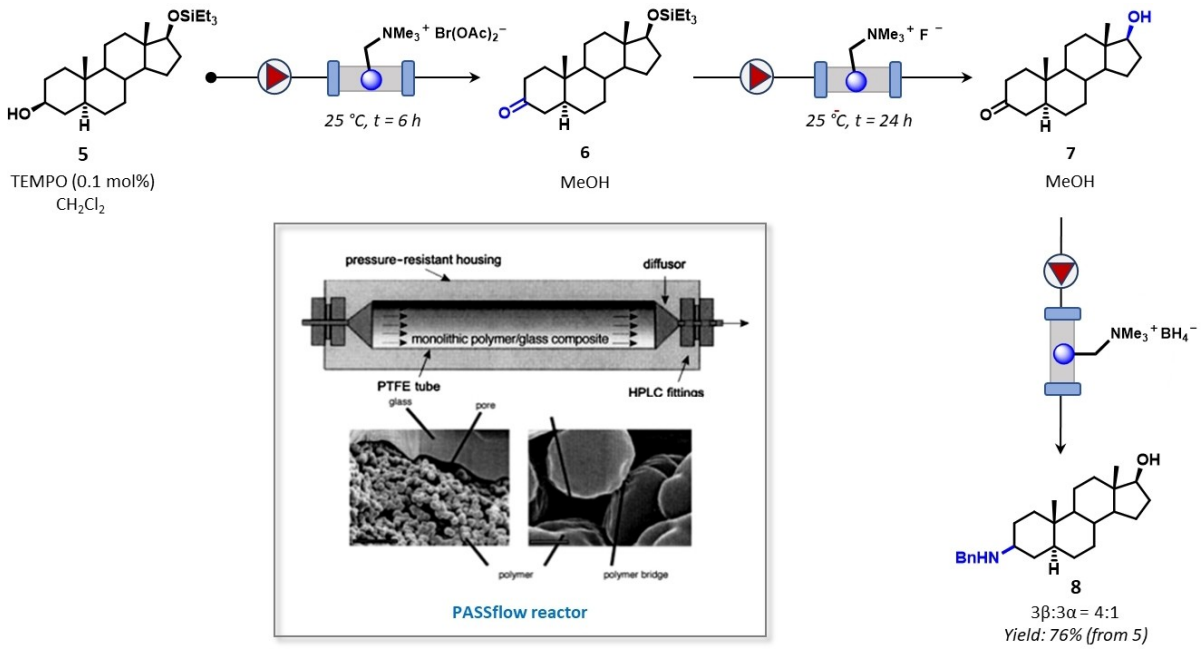
Figure 2. Typical synthetic set-up and potential benefits of continuous flow synthesis.

The basic principle of running a chemical process in a continuous flowing stream instead of a conventional round-bottomed flask is fascinating and offers several advantages.[11–12] These include the enhanced productivity and efficiency delivered through higher yielding reaction with fewer side products, the increased process automation and options of in- or on-line process control relating to reagent addition, mixing and heat transfer, particularly useful during reaction screening and optimization, as well as for the serial synthesis of compounds collections. Moreover, flow systems offer the possibility to create reaction conditions not easily achievable using traditional batch mode, and facilitate the integration with other enabling technologies such as the photo- and electro-chemistry, catalysis and biotransformations. The inherent safety benefits, resulting from the improved containment of hazardous reagents, reduced manual handling; on-time monitoring of risky processes have trust flow chemistry into the limelight as a modern and sustainable chemical tool.[13] In addition to safety, continuous flow technologies satisfy many of the concepts and requirements embodied by green chemical synthesis including minimal reagent consumption, prevention of waste generation, energy efficiency, improved throughput and catalyst reusability.

3. Multistep Flow Synthesis of Steroids

A multistep flow synthesis occurs when more than one transformation is conducted into a mobile motif in a single sequence.[14] Unlike batch reaction telescoping (multiple transformations without purification of intermediates), continuous flow approaches permit in-line work-up and purification, and reagent loading is accomplished at set points in the continuous flow sequence.[15] In this regard, continuous flow multistep synthesis can be viewed as a machine that mimics the biological processes in realizing complex natural products. Plug flow conditions and reaction compartmentalization is generally employed to achieve higher performance. Using such an approach, the reaction fluid moves through the reactor in thin uniform plugs and in vertical direction guaranteeing a better mixing. Accordingly, reactions are compartmentalized to each plug of fluid moving through the reactor limiting diffusion from one plug to another.

One of the pioneering attempts to apply flow chemistry for the manipulation of steroids dates back to 2001 when Kirschning and his team described the use of polymer-assisted solution-phase synthesis in the flow-through (PASSflow) reactor technology to prepare 3-amino-steroids (Scheme 2).[16] The system consisted in a monolithic microreactor containing a functionalized, highly porous polymer/glass composite.

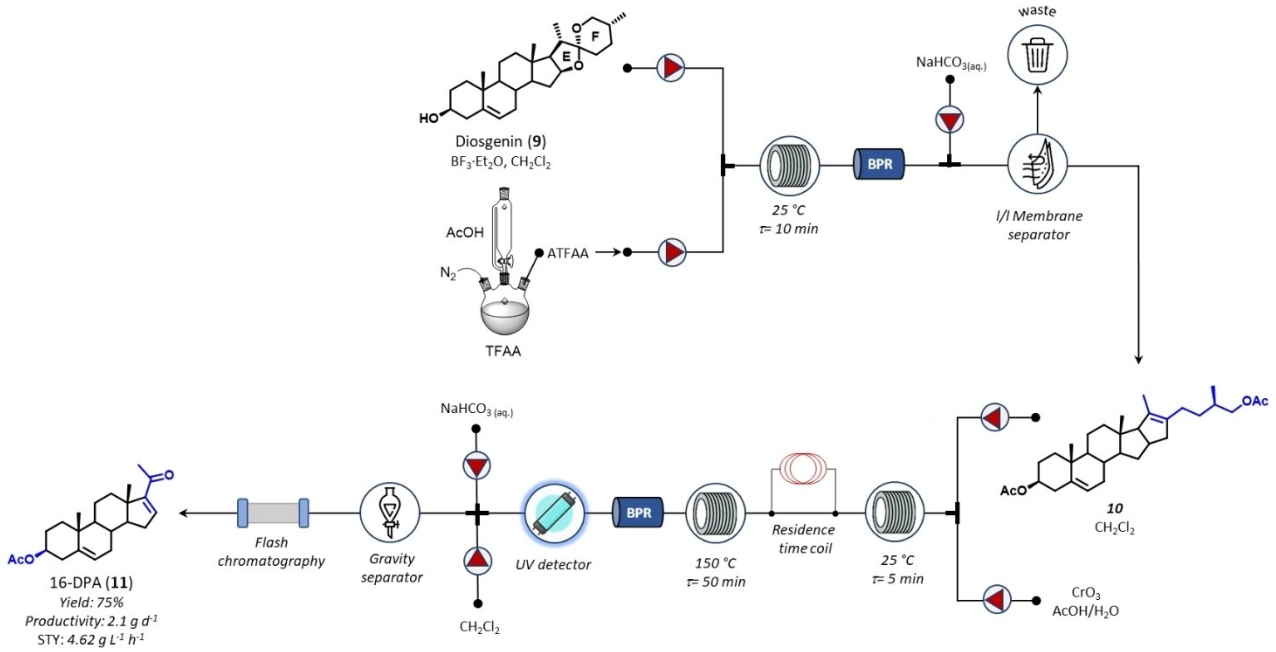


Scheme 2. Three-step continuous flow synthesis of the 3 *N*-benzylamino-17β-androsterol (**8**) using functionalized monolithic polymer/glass composites (PASSflow approach).

The cross-linked polyvinylchlorobenzene-divinylbenzene polymer was prepared by precipitation and polymerization inside the pore volume of highly porous glass rods with the aim to prevent reactor clogging or leaching. The functionalized rods were embedded into a polytetrafluoroethylene (PTFE) tube, which was then encapsulated using a pressure resistant housing reinforced with an epoxy resin. Three different PASSflow reactors were tested for the three-step synthesis of 3 *N*-benzylamino-5α-androstan-17β-ol (**8**). In particular, a 0.0125 M stock solution of **5** in dry CH_2Cl_2 containing a catalytic amount of TEMPO (0.1 mol%) was pumped under argon stream for 6 h through the PASSflow reactor filled with the bromate polymer-bound anion to afford the 3-oxosteroid **6**, in nearly quantitative yield. Then, a solution of **6** in MeOH was flowed through a fluoride loaded tubular reactor to give dihydrotestosterone (**7**) (90% yield), which was readily reacted with benzylamine and submitted to the reductive amination using a borohydride-based microreactor furnishing the desired steroid **8** in 76% yield (3β/3α = 4:1) (Scheme 2). In addition to reaction efficiency, the use of this flow-through technology permits the simple regeneration of the reactor system reducing costs and waste. Unfortunately, the necessity of solvent change (from CH_2Cl_2 to MeOH) requires out-flow operations making the synthesis discontinuous.

A more recent example of multistep flow synthesis was reported by us for the preparation of 16-dehydropregnenolone acetate (16-DPA, **11**), a fine chemical and a key synthon for the production of several natural steroids, sex hormones and drugs.[17] The method consisted in the Marker's degradation of a readily available and low cost sapogenin, namely diosgenin (**9**) (Scheme 3).

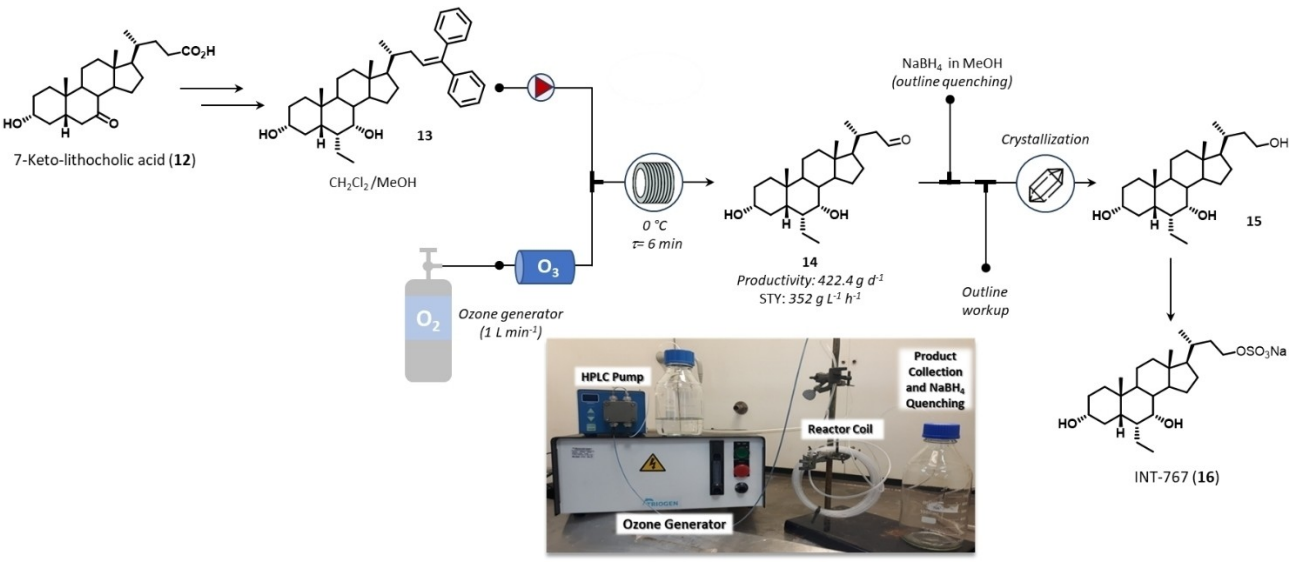
Traditional batch procedures involve the use of autoclave, long reaction times, harsh reaction conditions such as elevated temperature and pressure, which strongly affect the sustainability of the process and the product quality. To overcome these limitations, a three-step telescoped continuous flow synthesis was developed according to Scheme 3. A preliminary batch screening for the acetylation/acetolysis step allowed to identify acetic trifluoroacetic anhydride (ATFAA) as the acetyl source in the presence of a stoichiometric amount of $BF_3 \cdot Et_2O$ as the Lewis acid. The first step was conducted by mixing a solution of diosgenin (**9**) and $BF_3 \cdot Et_2O$ (2.0 equiv.) in CH_2Cl_2 with ATFAA (11 equiv.) prepared by reaction of trifluoroacetic anhydride (TFAA) with AcOH (Scheme 3). The resulting mixture was reacted into a 10 mL coil reactor maintained at 25 °C with a total flow rate of 1.0 mL min^{-1} ($\tau = 10$ min). The pseudodiosgenin-3,26-diacetate (**10**) (0.05 M in CH_2Cl_2) thus formed (80% yield) was submitted to oxidative cleavage by reaction with CrO_3 (0.07 M in $AcOH/H_2O$) using a 10 mL coil reactor at 25 °C with a total flow rate of 2 mL min^{-1} ($\tau = 5$ min). The reaction mixture was collected into a residence time reactor before entering a 10 mL coil reactor heated to 150 °C. A UV detector placed at the downstream of the flow reactor allowed reaction



Scheme 3. Telescoped flow set-up for the Marker degradation of diosgenin (**9**) to yield 16-dehydropregnenolone acetate (16-DPA, **11**).

monitoring and product detection. After 50 min the reaction outflow was combined with a 0.5 M aqueous solution of $NaHCO_3$ and CH_2Cl_2 to obtain 16-DPA (**11**) in 95% yield over two steps. The method was endowed with several advantages and improvements including a higher efficiency (productivity = 2.1 g d^{-1}, space time yield = 4.62 g L^{-1} h^{-1}) and overall yield (75%) under milder reaction conditions, the use of lower amounts of reagents, reduced manual handling, and simple downstream procedures, which resulted in high-quality material and safety standards.

Flow chemistry can be easily integrated into multi-step synthesis, also in combination with batch steps to facilitate the conduction of critical reactions. In 2022, we described the scale-up synthesis of the bile sulfate derivative INT-767 (**16**) (Scheme 4), an FXR/TGR5



Scheme 4. Combined batch/flow synthesis of INT-767 (**16**) with focus on the telescoped one-pot two-step oxidative cleavage procedure using an assembled flow ozonolysis device.

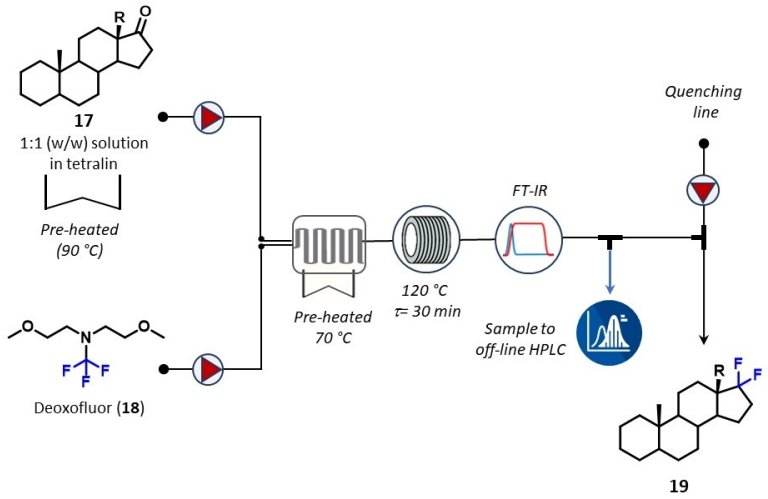
dual agonist with remarkable therapeutic potential for liver disorders.[18] Critical bottlenecks such as the side chain degradation were solved using flow technology. In particular, a telescoped one-pot two-step procedure was performed in a continuous mode without solvent switch using a readily assembled flow ozonolysis device (Scheme 4). The use of flow reactors facilitates the conduction of gas-liquid reactions that are deemed to be either too dangerous to be conducted at large scales in batch or not efficient due to the contact time of high-pressure requirements of the gases.[19] Thus, the ozone stream generated from molecular oxygen contained into a pressurized reservoir was mixed at 1 L min^{-1} with a 0.12 M stock solution of the *bis-nor*-5β-cholanyldiphenylethylene derivative (**13**) in CH_2Cl_2/MeOH (1:1, v/v) pumped at 1 mL min^{-1} (Scheme 4). Aldehyde **14** was obtained with a productivity of 422.4 g d^{-1} and a space time yield of 352 g L^{-1} h^{-1}. The resulting liquid-gas biphasic system was flowed through a reactor coil cooled at 0 °C (τ = 6 min).

The output was collected into a round bottom flask in the presence of a stirring solution of $NaBH_4$ in MeOH at 25 °C, enabling both the quenching of ozone and the reduction of the C23-aldehyde group into the corresponding alcohol in excellent yields (88–90%) on multigram scale (Scheme 4). Remarkably, the ozonolysis reaction did not involve any competitive oxidation reaction at the hydroxyl groups at C3 or C7 positions ruling out the need of protection/deprotection extra-steps. This represents a great advantage to streamline the overall process as the formed intermediate **15** can be easily purified by crystallization from *t*butyl methyl ether (TBME), avoiding time-consuming and expensive chromatography.

4. Steroid Synthesis Optimization by Computer-Assisted Flow Systems

The integration of flow chemistry with statistical design of experiments (DoE) is a valuable approach to develop and optimize synthetic processes towards relevant building blocks and drugs.[20] Statistical designs offer a greater insight into method optimisation and a better understanding of the chemical process than the commonly used one-factor-at-a-time (OFAT) approach, in which experiments are planned by scientific intuition fixing all experimental parameters except for one. Indeed, OFAT disregards any synergistic effects between any parameter in the multi-dimensional and complex chemical space and, therefore, it does not consider the effect of interactions between parameters. Optimization by experimental design incorporates statistical algorithms, mathematical models, predicting tools, feedback control, and validation to generate new optimal conditions. Once the experiments are performed, the resulting data can be fitted into a regression equation and used to find optimum reaction conditions. Continuous flow technology is ideally suited for this scope as the integration of in/on-line monitoring is relatively easy, reaction parameters can be tightly controlled and fine-regulated.[21] Moreover, labor savings are determinant, especially when computer-assisted flow systems operate in automated fashion accelerating data output and analysis.

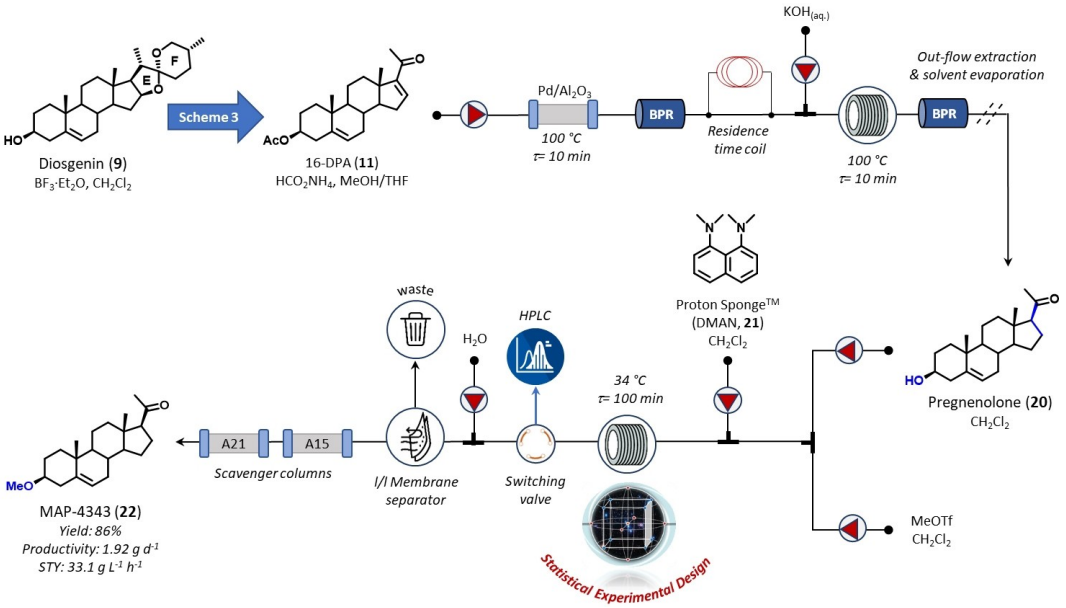
In 2008, researchers at Bayer Schering Pharma reported the development of a continuous flow process for the fluorination of steroids using bis(methoxyethyl)-aminosulfurtrifluoride (deoxofluor, **17**) (Scheme 5).[22] The fluorination was conducted on the 17-oxo steroid **7** by the preliminary investigation of the effect of reagent stoichiometry (1–3 equiv.) and



Scheme 5. Continuous flow *gem*-difluorination of 17-oxo steroids.

temperature (80–120 °C) on the reaction outcome. Reaction parameters, the rate constant and the activation energy were determined by Berkeley Madonna, a software developed at Berkeley (California, USA) to model complex dynamic systems, including chemical reactions of enzyme kinetics.[23] A high degree of correlation between the experimental and the simulated data was obtained, and the generated mathematical model was then interrogated to determine best conditions for continuous flow operations. The flow set-up consisted of two calibrated rotary piston pumps, a micromixer element, a perfluoroalkoxy (PFA) reactor tubing heated in an oil-bath, a back-pressure regulator (BPR), and was assisted by reaction monitoring based on in-line ATR FT-IR and an off-line HPLC analysis. Experiments were conducted by pumping two feed solutions: one containing the steroidal substrate **17** (1:1, w w^{-1} solution in tetralin) pre-heated at 90 °C and the second one containing deoxofluor (**18**). The optimal reaction conditions consisted in a residence time of 30 min, a temperature of 120 °C and the use of 3 equiv. of deoxofluor (**18**) (Scheme 5). Using this method, the 17-*gem*-difluoro steroid **19** was obtained in about 60% yield. Unfortunately, the lack of some data (e.g., volume reactor, processed material) does not allow to calculate productivity indices.

More recently, a central composite design (CCD) was employed for the synthesis optimization of the neurosteroid 3β-methoxypregnenolone (MAP4343, **22**) from pregnenolone (**20**) (Scheme 6).[24] CCD is a response surface design usually employed for the identification of optimal conditions and to study the impact of small experimental changes on the reaction outcome.[20] Initially, batch screenings led to the initial identification of the Proton Sponge™ (DMAN, **21**) and methyl trifluoromethanesulfonate (MeOTf) as appropriate reagents for the flow reaction. Next, a set of experiments was performed with the aim to define the solvent system (CH_2Cl_2/MeCN), reagent stoichiometry (MeOTf = 3.5 equiv., **21** = 2 equiv.) and their relative concentrations. Reaction optimization was based on the investigation of temperature (T = 25–40 °C) and residence time (τ = 15–120 min). 11 experiments were conducted in a sequential, automated fashion using a flow set-up constituted by three pumps, a 10 mL coil reactor, a BPR, a UV detector, an autosampler, a fraction collector and a six-way switching valve connected to the HPLC equipment for yield determination and side-product profiling. The data acquired were fitted into quadratic equations furnishing the corresponding mathematical models that were submitted to analysis of variance (ANOVA). Optimum conditions (T = 34 °C; τ = 100 min) were adopted to scale-up the process with the integration of downstream devices as membrane liquid-liquid separators, scavenging columns and automated flash chromatography (Scheme 6). MAP4343 (**22**) was obtained in high purity grade and 86% yield (productivity = 1.92 g d^{-1}, space time yield = 33.1 g L^{-1} h^{-1}). Additional features of this approach included the use of MeOTf instead of more toxic methylating agents, a reduced handling of chemicals, reagent recovery and the mild experimental conditions that made the process with a safer imprint. Remarkably, this single step process was integrated within a multistep sequence enabling the continuous flow synthesis of MAP4343



Scheme 6. DoE-assisted optimization of the neuroactive steroid MAP4343 (**22**) and integration into the multistep flow synthesis from diosgenin (**9**).

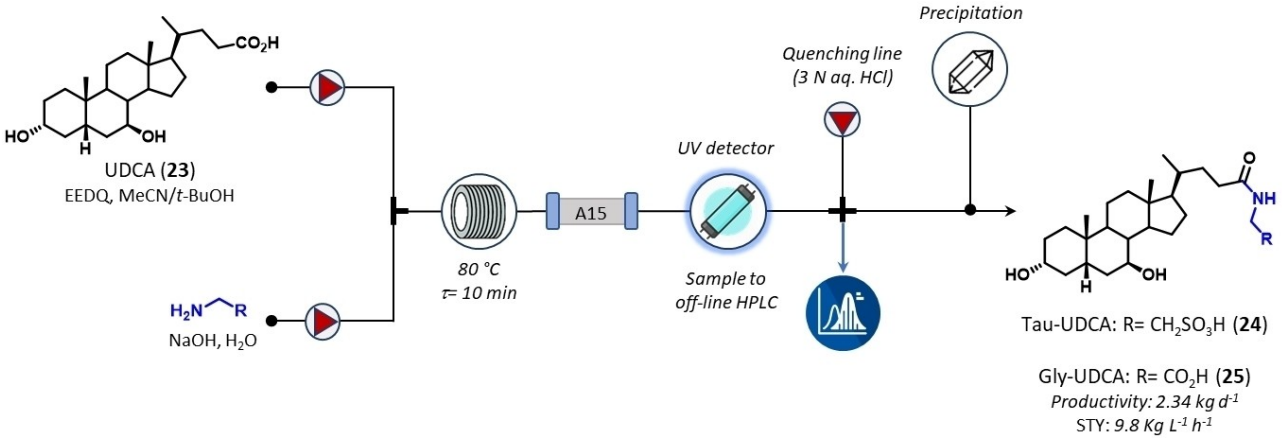
(**22**) from diosgenin (**9**) at laboratory scale with an overall yield of 64% over six steps (Scheme 6).

5. Bile Acid Functionalization by Flow Chemistry

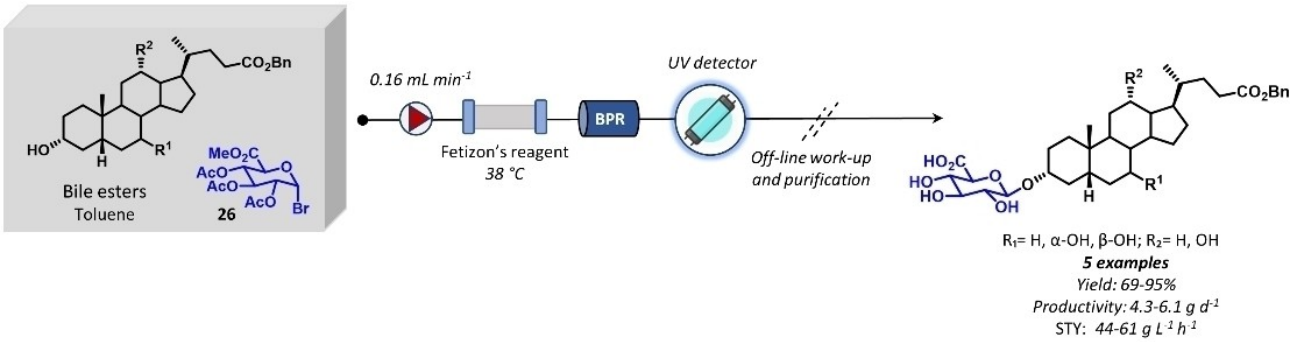
Bile acids are a peculiar class of acidic amphipathic steroids that have found major therapeutic applications for treating metabolic and inflammatory diseases affecting tissues such as the liver, gallbladder and intestine.[25] The ready availability of bile acid metabolites is therefore of crucial importance to determine their levels in urine and plasma as biomarkers for clinical diagnosis and response to therapy. Conventional methods for preparation of bile acid metabolites include extractive methodologies and isolation from biological fluids, enzymatic preparations, and chemical syntheses. Whilst the use of enzymatic and extractive methodologies remains elusive, the chemical synthesis suffers from several drawbacks including low yields, the use of drastic reaction conditions, long reaction times and tedious purifications. Furthermore, extra steps of protection-deprotection are generally required in order to avoid byproduct formation.

In 2012, we described the flow-assisted *N*-acyl amidation of human bile acids to give tauro- and glyco-conjugates, the major bile acid metabolites (Scheme 7).[26] Ursodeoxycholic acid (UDCA, **23**) and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) were selected as the model substrate and the activating agent, respectively. The flow experiments were performed in a modular flow equipment endowed with a two-loop injection system, two HPLC pumps, a 10 mL thermo-coupled reactor coil, a BPR, a UV detector and fraction collector (Scheme 7). This set-up was useful to conduct a series of experiments to evaluate the effect of the base, the solvent system, the temperature and the residence time. Reactions were performed by loop injection of a 0.2 M organic solution of **23** and EEDQ (2 equiv.) and an aqueous solution of glycine or taurine (2.5 equiv.) in the presence of 2 equiv. of base. After the injection, the two solutions were mixed in a T-junction, pumped at the desired flow rate through the coil reactor, and heated at the selected temperature. The output was monitored by a UV-detector, collected and analyzed by calibrated ELSD-HPLC to determine conversion yield and product purity. The best conditions were obtained at 80 °C, with a residence time of 10 min, using H_2O/MeCN/*t*BuOH (4:3:1, v/v/v) as the solvent system and NaOH as the base of choice, to provide conjugates **24** and **25** in 95% and 91%, respectively. The reaction with glycine was successfully scaled-up and the crude was purified by Michel-Miller chromatography column packed with Amberlyst A-15 to remove the excess of amino acid and part of the quinolone released from EEQD. Accordingly, glyco-UDCA (**25**) was obtained in 91% yield (productivity = 2.4 g h^{-1}, space time yield = 9.8 kg L^{-1} h^{-1}) and excellent purity (98%) by simple precipitation (Scheme 7). The method was finally applied to major human bile acids obtaining the corresponding conjugates in good to high yield (75–95%).

In 2018, the selective C3 glucuronidation reaction of bile acids was reported to achieve important probes and gain insights into the bile acid signalling and detoxification pathways (Scheme 8).[27] The desired products were synthesized by Koenigs-Knorr using a tubular reactor packed with the Fetizon reagent.[28] The reaction was optimized by exploring key variables such as the temperature (25–80 °C), flow rate (0.1–0.4 mL min^{-1}) and stoichiometry of glucuronyl donor **26** (1.2–3 equiv.). A set of 14 experiments and five replicates at the central point was instrumental to find the best experimental conditions (**26** = 2.6 equiv., flow



Scheme 7. Continuous flow synthesis of taurine- (Tau-UDCA) and glycine- (Gly-UDCA) conjugated ursodeoxycholic acid derivatives.



Scheme 8. Regioselective continuous flow glucuronidation of bile acids.

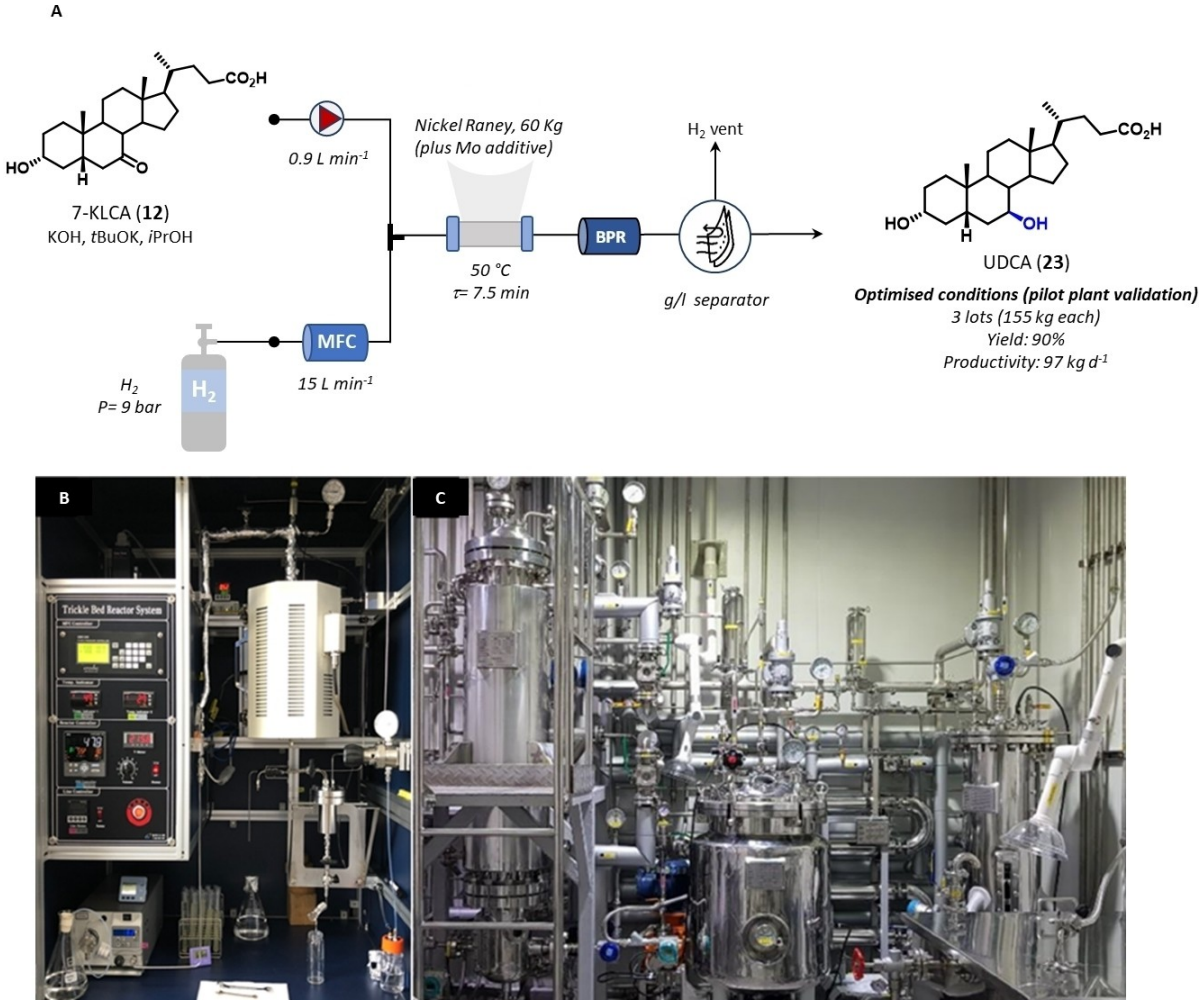
rate = 0.16 mL min^{-1}, T = 38 °C). After the injection and the switching of the valve through the loop, the solution of the bile acid benzyl ester (0.1 M) and the glucuronyl donor **26** in toluene was flowed through the Fetizon-based reactor reagent heated at the selected temperature (Scheme 8). After reverse-phase chromatography, the glucuronyl conjugates of human bile acids were isolated in good to excellent yields (69–95%, productivity = 4.3–6.1 g d^{-1}, space time yield = 44–61 g L^{-1} h^{-1}) and high selectivity. Unfortunately, Remarkably, reactions proceeded with a high degree of regioselectivity, as no C7- or C12-glucuronidation was detected, and chemoselectivity. Although Fetizon's reagent is a well-known oxidizing agent, the presence of 3-keto-derivatives was never observed in the crude reaction mixtures. The synthesized glucuronyl compounds were then evaluated in terms of properties and ability to modulate the FXR receptor.[27]

More recently, the continuous flow synthesis of UDCA (Ursodiol™) (**23**) was realized by catalytic hydrogenation of 7-keto-lithocholic acid (7-KLCA, **12**) (Scheme 9).[29] Different experimental parameters were screened to optimize the reaction conditions for pilot plant production. Authors found that Raney nickel in the presence of small amount (0.5–0.6%) of molybdenum in *i*PrOH was able to increase the conversion rate and selectivity ratio of the C7-carbonyl reduction. Best temperature range was at 40–50 °C while fixed bed reactors showed a better efficiency than the moving-bed type. The gram-scale lab experiment was conducted in a commercially available CCR-1000G instrument by reacting a 0.05 M *i*PrOH solution of 7-KLCA (**12**), KOH (1 equiv.) and *t*BuOK (0.5 equiv.), and at a flow rate of 0.06 mL min^{-1}. The stream of hydrogen was supplied at 40 °C using a mass-flow controller (MFC) (5 mL min^{-1}, 3.3 bar) to obtain UDCA (**23**) in 93% yield. Further optimization enabled the efficient scale-up (up to 155 Kg) with high yield (90%) and a productivity of about 4 Kg h^{-1} (97 kg d^{-1}) (Scheme 9). Overall, this flow approach was superior to conventional methods in terms of yield, selectivity, productivity and safety due to the higher interfacial area of flow devices essential for the efficient mass transfer rate.

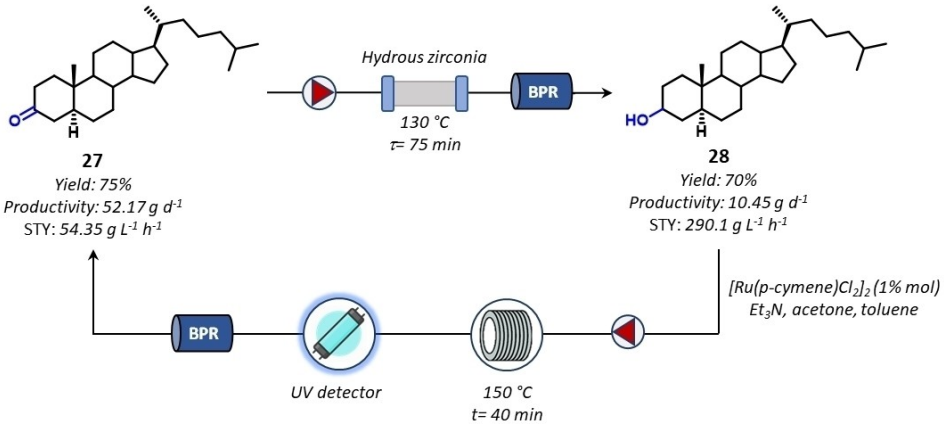
Along this line, the bile acid precursor 3-hydroxy-dihydrocholesterol (**28**) was prepared by the Meerwein-Ponndorf-Verley reduction of 3-keto-dihydrocholesterol (**27**) (Scheme 10).[30] The method described by S. Ley team to reduce carbonyl compounds consisted in the use of a packed bed reactor filled with hydrous zirconia and heated at 130 °C. Accordingly, **27** was successfully reduced in 70% isolated yield (τ = 75 min, productivity = 10.45 g d^{-1}, space time yield = 290.1 g L^{-1} h^{-1}). Later, the same group studied the Oppenauer oxidation by ruthenium-catalyzed hydrogen-transfer reaction.[31] The flow set-up comprised a HPLC pump, a 40 mL stainless-steel heated reactor coil, a BPR and an in-line UV detector. Dichloro(*p*-cymene)ruthenium(II) dimer (1 mol%) was chosen as a commercially available and low cost catalyst. Thus, 3-hydroxy-dihydrocholesterol (**28**) was oxidized at 150 °C to the corresponding 3-keto-dihydrocholesterol (**27**) in 75% isolated yield (productivity = 52.17 g d^{-1}, space time yield = 54.35 g L^{-1} h^{-1}) using Et_3N (2 equiv.) and a solution of acetone/toluene (8:2, v v^{-1}), which guaranteed the complete solubility of the steroidal substrate (Scheme 10). Worth of note, both processes employed easy-to-make and reusable tubular flow reactors that in addition to be unexpensive, avoid time-consuming work-up procedures.

6. Flow Photochemistry and Steroids

The combination of flow technology with photochemistry benefits from better and more uniform irradiation compared to batch conditions resulting in faster and more selective reactions.[32] Moreover, flow reactors can be easily pressurized providing good mass transfer and large interfacial areas without compromising safety or irradiation. Low-energy, tunable light sources are favored in combination with suitable cooling devices to effectively control both temperature and energy input. Finally, continuous flow reactors allow simple scale-up of photoreaction without alteration of



Scheme 9. A) Flow set-up for the packed-bed hydrogenation of 7-keto-lithocholic acid (**12**) to ursodeoxycholic acid (Ursodiol™) (**23**). B) Lab scale-up flow system. C) Pilot production flow set-up.

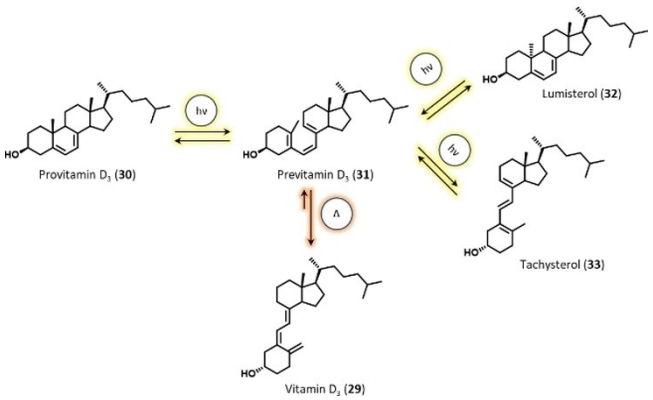


Scheme 10. Zirconia-catalyzed Meerwein-Ponndorf-Verley reduction of 3-keto-dihydrocholesterol (**27**) and ruthenium-catalyzed Oppenauer oxidation of 3-hydroxy-dihydrocholesterol (**28**) under flow conditions.

reaction parameters, and the short residence times within the photoreactor reduce decomposition of the substrate or the product due to over-irradiation. The steroid derivative, vitamin D_3 (**29**) can be prepared by

photo-isomerization of provitamin D_3 (**30**) to previtamin D_3 (**31**), followed by the thermal isomerization reaction (Scheme 11). However, the current industrial scale production of vitamin D_3 (**29**) suffers from several drawbacks including the low yield (≤20%) and selectivity of the photochemical isomerization that causes the formation of side products (e.g., lumisterol (**32**) and tachysterol (**33**)), which are difficult to remove and require several crystallization operations.

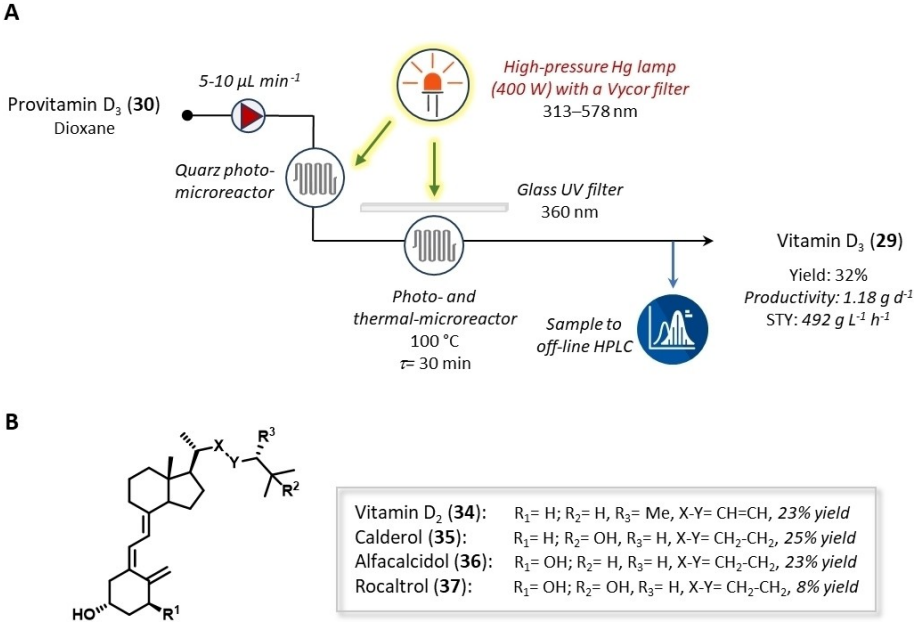
In 2010, the two-step continuous flow synthesis of vitamin D_3 (**29**) from provitamin D_3 (**30**) was reported (Scheme 12).[33] A high-pressure mercury lamp was used as high-intensity economical light source to perform the simultaneous photo- and thermal-reaction in absence of any laser emission source, sensitizer or filter compounds. The flow apparatus consisted of two microreactors connected through PEEK tubing. The first quartz microreactor (250 mm length, 200 µm depth, 1 mm width, 50 µL volume) was irradiated at 313–578 nm using a 400 W high-pressure mercury lamp with a Vycor filter. Without any purification, the outflow stream from the first reactor was pumped into a second quartz microreactor (500 mm length, 200 µm depth, 1 mm width, 50 µL volume) irradiated at 360 nm and heated at 100 °C.



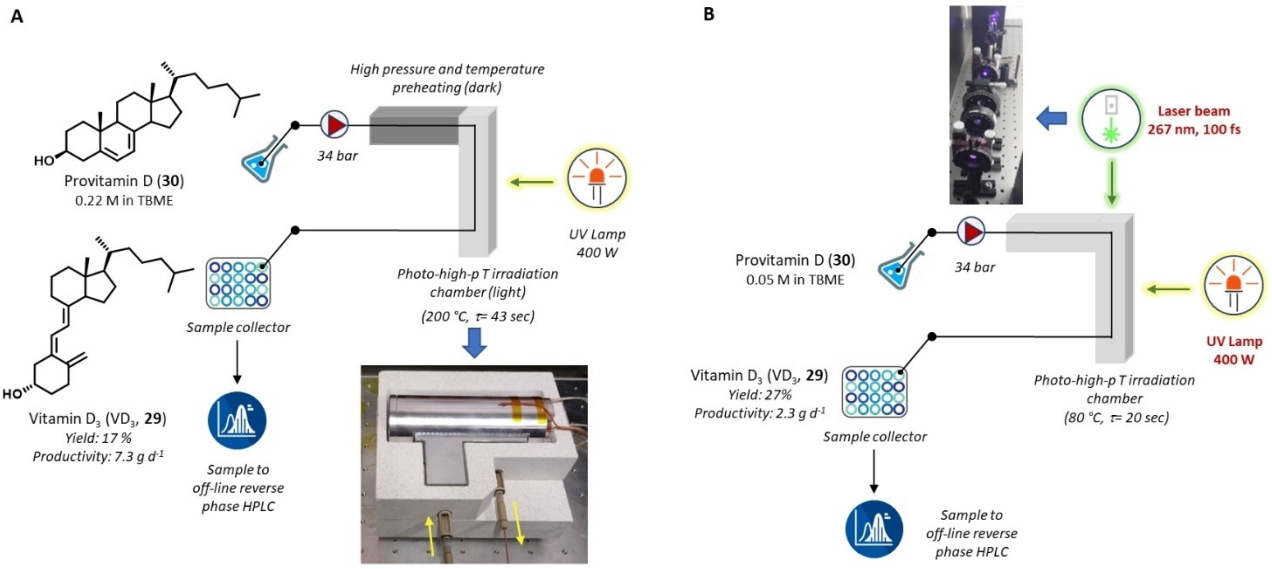
Scheme 11. Photosynthesis of vitamin D_3 (**29**) and related side products.

After the preliminary screening aimed at defining the best solvent, substrate concentration and residence time, the desired vitamin D3 (**29**) was obtained in good yield (HPLC yield = 60%, isolated yield = 32%, productivity = 1.18 g d^{-1}, space time yield = 492 g L^{-1} h^{-1}) by pumping a 30 mM solution of provitamin D3 (**30**) in dioxane at 5 µL min^{-1} (τ = 30 min) (Scheme 12). The same flow system was utilized for the preparation of four bioactive vitamin D analogues including vitamin D_2 (calciferol, **34**), 25-OH vitamin D_3 (calderol, **35**), 1α-OH vitamin D_3 (alfacalcidol, **36**), and 1α,25-$(OH)_2$-vitamin D_3 (rocaltrol, **37**) (Scheme 12).[34]

In 2018, Hessel and co-workers described the use of a photo-high pressure and temperature microreactor under strictly controlled conditions (Scheme 13).[35] In this set-up, a solution of **30** in TBME was pumped through a pre-heating dark chamber, and then in high pressure/temperature irradiation chamber made of reflective aluminium in order to enhance internal light reflections generated from the mercury lamp used as the light source (Scheme 13A). Under optimized conditions ([**30**] = 50–220 mM, τ = 43 s, P = 34 bar,



Scheme 12. A) Flow set-up for the photosynthesis of vitamin D_3. B) Vitamin D_3 analogues **34**–**37** prepared using the optimized flow conditions.

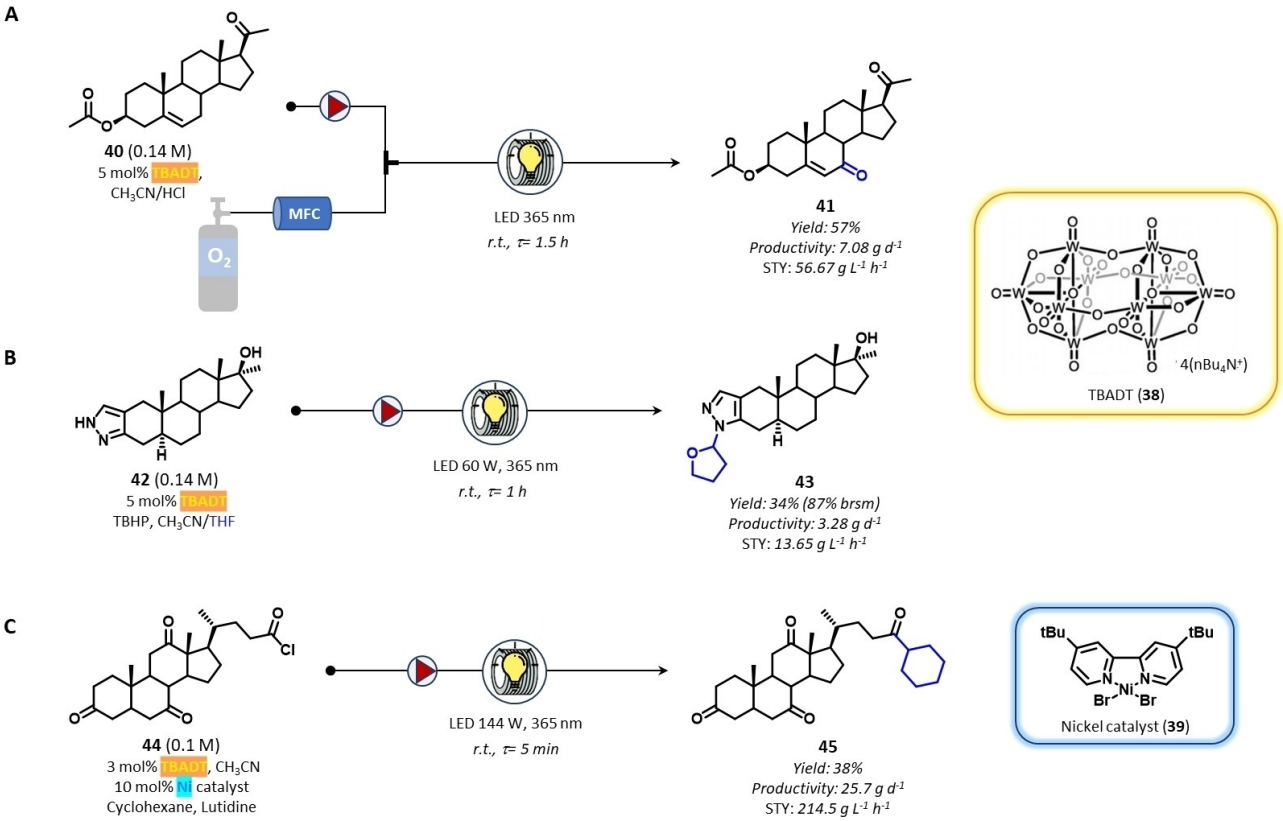


Scheme 13. Vitamin D_3 photosynthesis under flow conditions using the high p-T reactor.

T = 200 °C), the reaction gave 42% conversion yield including vitamin D_3 (**29**) (17%) and intermediates (25%), and the productivity increased by up to two orders of magnitude (7.3 g d^{-1}) with respect to the previous flow synthesis.[36] Later, the same team improved the method by using a laser beam resulting in a higher conversion rate (66%) albeit lower productivity (2.3 g d^{-1}) (Scheme 13B).[37] Overall, the combination of flow microreactor technology with high-intensity and economical light sources can be an effective tactic to improve both the product yield and purity without requiring purification extra-steps or high-dilution conditions.

Recently, Noël group employed the potential of flow photocatalysis for several transformations using tetrabutylammonium decatungstenate (TBADT, **38**) as hydrogen atom transfer (HAT) agent for C–H aerobic oxidation (Scheme 14).[38] The flow set-up comprised a continuous flow photomicroreactor consisting of a PFA capillary reactor (750 µm ID, 5 mL) and a LED irradiation (365 nm). A syringe pump was used to deliver the stock solution of the substrate (0.14 M in CH_3CN/1 M HCl, 2.5:1, v v^{-1}) and **38** (5 mol%) that was combined with a stream of dioxygen delivered by a MFC at atmospheric pressure. Worth of note, within the substrate scope that included 16 activated and 14 unactivated substrates, the allylic oxidation of pregnenolone acetate (**40**) at the C7 position was achieved in 57% yield (τ = 1.5 h, productivity = 7.08 g d^{-1}, space time yield = 56.67 g L^{-1} h^{-1}) (Scheme 14A). More recently, the same research group combined the decatungstate-mediated HAT photocatalysis with the oxidative radical-polar crossover for the oxidative C(sp^3)–H heteroarylation as a late-stage functionalization (LSF) strategy.[39] After batch screening, the reaction was successfully translated and optimized in a UV-150 (λ = 365 nm, power = 60 W, ID PFA tubing = 1.3 mm, V = 10 mL) flow apparatus. Within the investigated substrate scope (40 examples), the LSF of the anabolic steroid stanozolol (**42**) was achieved under optimized conditions ([**42**] = 0.2 M in CH_3CN, 5 mol% TBADT, 3 equiv. TBHP, 18 equiv. THF) to provide compound **43** in 34% yield (τ = 1 h, productivity = 3.28 g d^{-1}, space time yield = 13.65 g L^{-1} h^{-1}) (Scheme 14B). The same authors reported the TBADT photocatalyzed acylation/arylation of unfunctionalized alkyl derivatives under flow conditions.[40] The method exploited the ability of the decatungstate anion to act as a hydrogen atom abstractor and produce nucleophilic carbon-centered radicals that are intercepted by a nickel catalyst (**39**) for building novel C(sp^3)–C(sp^2) bonds (Scheme 14C). Reactions were performed using a microreactor (5 mL, 0.76 µm ID) where the stock solutions of the reactants were pumped at 1 mL min^{-1} of flow rate (τ = 5 min) and irradiated by a LED source (365 nm, 144 W).

Under the optimized conditions (TBADT 3 mol%, Nickel catalyst 10 mol%, 1.1 equiv. lutidine, 5 equiv. cyclohexane), derivative **45** was obtained in 38% isolate yield (productivity = 25.7 g d^{-1}, space time yield = 214.5 g L^{-1} h^{-1}) from the corresponding dehydrocholic acyl chloride **44** (Scheme 14C). Altogether these studies demonstrated that the aerobic oxidation of C(sp^3)–H bond *via* hydrogen atom transfer under mild conditions represents a powerful chemical transformation and can be applied as LSF strategy in the functionalization of relevant steroidal products.



Scheme 14. TBADT-catalyzed HAT of steroidal scaffolds under flow conditions.

7. Biotransformations of Steroids Under Flow Conditions

Up to date, flow chemistry has been mainly applied to perform classical organic chemistry reactions, metal- and organometal-catalyzed reactions, and photochemical and photocatalytic reactions. However, the combination between flow chemistry and biocatalysis can substantially contribute to the transition towards a more sustainable manufacturing of fine chemicals and APIs, as recently highlighted by the IUPAC.[41] This potential stems from the versatility of flow chemistry technology and the impressive and fast developments in the field of biocatalysis over the past two decades. Nowadays, the discovery of new enzymes *via* bioinformatics and metagenomics approaches, as well as the engineering of enzymes by directed evolution or rational (e.g., structural-guided) strategies have reached a level of maturity that make them powerful and reliable tools for the rapid laboratory search, development and improvement of available and new catalytic activities.[42] On the one hand, despite the recent progress in the field of biocatalysis in flow[43] its implementation for the chemo-, regio- and stereoselective synthesis of steroids is underdeveloped. On the other hand, much of the recent research in biocatalytic conversion of steroids was conducted in batch, thus generally leading to moderate productivities.[44] One main limitation is the current unavailability or difficult accessibility for well-characterized enzymes (e.g., oxidoreductases, transferases) that can operate chemical functionalization of diverse steroidal backbones with complementary regio- and stereo-selectivity. In fact, most of the publications in flow report on immobilized whole cells that are used as "black-box" biocatalysts. The alternative is the use of "classical" and commercially available enzymes such as hydrolases. Flow chemistry is commonly combined with whole cells biocatalysts for the biocatalytic synthesis of steroids. This solution allows for lowering the production costs due to the avoidance for isolation or purification of the enzyme, the immediate applicability, and the possibility to harness the native cellular reaction environment.[45] This last feature often results in higher biocatalyst stability and avoidance of addition of cofactors (e.g., NAD^+ for dehydrogenases).

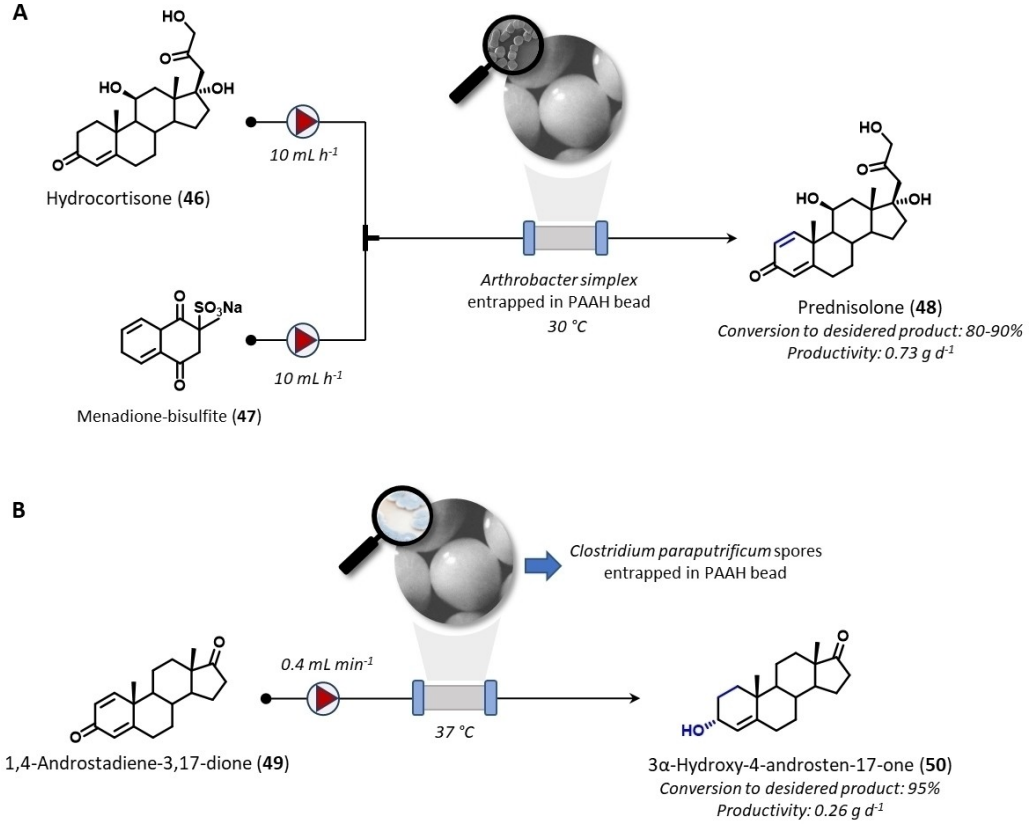
However, the advantages of using whole-cell biocatalysts come at the expense of significant drawbacks such as mass transfer limitation due to difficult cell membrane permeability, increase possibility for side-reaction due to the presence of other enzymes and biomolecules in the cells, and more complicated downstream processing. Therefore, a gradual transition from

the application of whole-cell biocatalysts to recombinant enzymes, even immobilized, has been observed in recent times. This progress has brought many advantages such as a higher reaction selectivity and purity of the final product (that is critical for API manufacture),[43b,d,g] a facilitated mass transfer in a flow reactor, and a higher biocatalyst density on the carrier material. These advantages well compensate for the need of supplementing a cofactor, or a possible increase of production cost, or reduced stability. However, these last two foreseeable drawbacks of isolated enzymes can be alleviated or even completely solved by efficient enzyme immobilization,[46] and protein engineering.[47] A proper choice of the carrier material is fundamental for immobilization as it must ideally have a large surface area, sufficient functional group for anchoring the enzyme, hydrophilic character, water insolubility (when working in an aqueous buffer), and resistance to enzymatic degradation, besides the general common requirements for flow chemistry such as chemical and thermal stability, mechanical strength and recovery.[48] In this section, we describe a selection of biocatalytic processes for the functionalization or manipulation of steroids with an emphasis on current strength and limitations in relation to the above-mentioned parameters.

7.1. Biotransformations with Dehydrogenases and Oxidases

One of the first examples of flow-assisted biocatalytic transformation of steroids was reported by Freeman *et al.* in 1988. It concerned the continuous Δ^1-dehydrogenation of hydrocortisone (**46**) to prednisolone (**48**) using immobilized whole cells of *Arthrobacter simplex* (Scheme 15A).[49] Microbial immobilization by entrapment into polyallylamine hydrazide (PAAH)[50] enabled to increase enzyme lifetime up to 30–40 days of continuous operation. Ethylene glycol was added as the co-solvent to improve the solubility of **46**. Notably, the difference of K_M values between free and immobilized enzyme was negligible (0.48 mM *vs* 0.83 mM, or 1.28 mM *vs* 1.46 mM at 10% or 20% v v^{-1} of ethylene glycol as cosolvent). These observations revealed limited diffusional limitations upon enzyme immobilization.

Menadione sodium bisulfite (MBS, **47**) was applied as an artificial electron donor to overcome the scarce solubility of molecular oxygen in the aqueous buffer containing the organic cosolvent, and the consequent decrease in the reaction rate. The continuous process was performed in a plug-flow reactor, containing the PAAH-bead entrapped *Arthrobacter simplex* cells, that



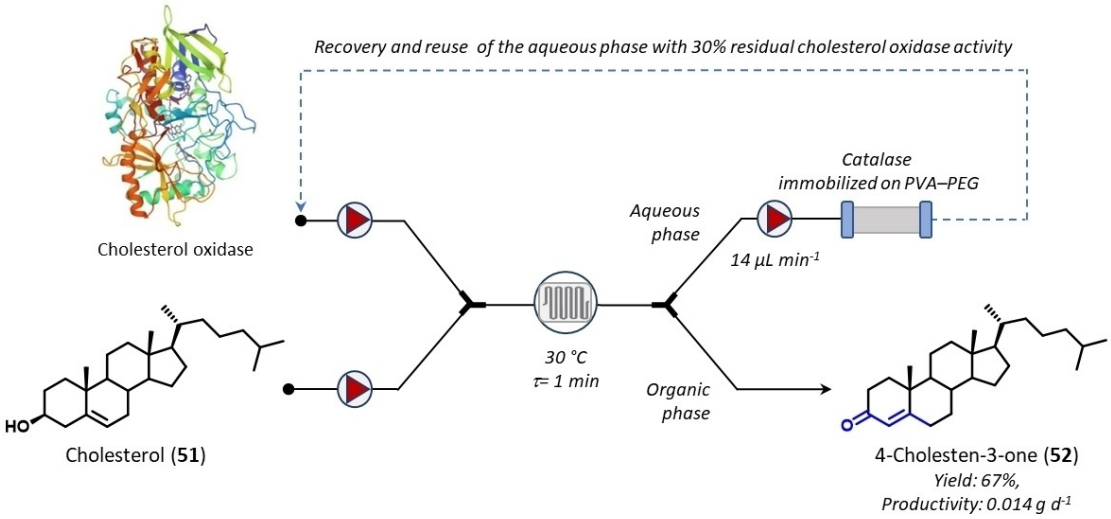
Scheme 15. Continuous stereospecific reduction of A) hydrocortisone (**46**) to prednisolone (**48**) and B) 1,4-androstadiene-3,17-dione (**49**) to 3α-hydroxy-4-androsten-17-one (**50**) by PAAH bead-entrapped enzyme spores.

was thermostated at 30 °C. In presence of equimolar amounts of MBS (2.5 mM in Tris buffer), an initial conversion of 50% was achieved but this value fell over the time due to MBS autooxidation.

The replacement of Tris with phosphate buffer (50 mM) and the feeding of **47** from a separate reservoir stored at room temperature in dark conditions solved the issue and enabled the efficient bioconversion of hydrocortisone (**46**). Therefore, the aqueous solution of **46** (4.4 mM in 20% v v^{-1} of ethyleneglycol) and MBS (**47**) (12 mM in 50 mM phosphate buffer) were reacted with a total flow rate of 20 mL h^{-1}, and reached a stable conversion (yield = 80–90%, productivity = 0.73 g d^{-1}) over 40 days using 2.8 g (w w^{-1}) of PAAH-bead entrapped cells (cell loading: 14 g kg^{-1} gel). Remarkably, neither cell leakage nor PAAH-bead deterioration were observed throughout the experiments (Scheme 15A).

Similarly, two years later S. Abramov and collaborators reported the continuous anaerobic stereospecific Δ^4-3-keto-steroid reduction using PAAH bead-entrapped *Clostridium paraputrificum* spores (Scheme 15B).[51] Since *Clostridium* species generally require anaerobic growth conditions, the spore suspension of the microorganism in sterile PAAH solution was used as the bead core. The presence of ethylene glycol (10%) as water-miscible co-solvent improved both the 3-keto-Δ4-steroid solubility and its diffusion; additionally, it served as a carbon source for cells thereby influencing cell growth and sporulation. Since consecutive batchwise operations showed low efficiency due to the cell metabolic state, a sterilized column packed with PAAH bead-entrapped *Clostridium paraputrificum* spores was tested for continuous flow biocatalysis. Under these conditions, the conversion of 1,4-androstadiene-3,17-dione (**49**) to 3α-hydroxy-4-androsten-17-one (**50**) reached a steady state after 20 h. A 95% conversion yield was maintained for 100 h (productivity = 0.26 g d^{-1}), whereas a decrease of yield (ca. 30%) per incubation was observed in repeated consecutive bioconversions (5 h each) (Scheme 15B).

More recently, the use of microreactor technology has gained consideration in the field of flow biocatalysis. For instance, Marques and coworkers reported the continuous production of 4-cholesten-3-one (**52**) (Scheme 16).[52] To this aim, an O_2-saturated solution of cholesterol (**51**) in *n*-heptane (2.6 mM, 10–154 µL min^{-1}) and an air-saturated phosphate buffer (50 mM, 5–72 µL min^{-1}) containing cholesterol oxidase (0.1 mg mL^{-1}) were separately fed to the inlets of a Y-shaped glass microchannel reactor thermostated at 30 °C (Scheme 16). For removing the hydrogen peroxide formed as the by-product that would cause the deactivation of cholesterol oxidase, the aqueous phase at the outlet of the microreactor was pumped (14 µL min^{-1}) through a plug-flow reactor containing immobilized catalase. The catalase effects the disproportion of hydrogen peroxide to dioxygen and water. This continuous integrated set-up was therefore compared with traditional reactors. The results showed that microchannel reactor achieved conversion yields of 67%, which are similar to those obtained using stirred tank reactors and mechanically-stirred jacketed batch reactors, but with a significant reduction of the residence time for the flow system (from 20 and 90 minutes in packed-bed and stirred tank reactor, respectively, to 1 minute using microreactor). Consequently, the flow system resulted in a higher productivity (0.014 g d^{-1}). Notably, the integration of cholesterol oxidation with the catalase plug-flow reactor allowed



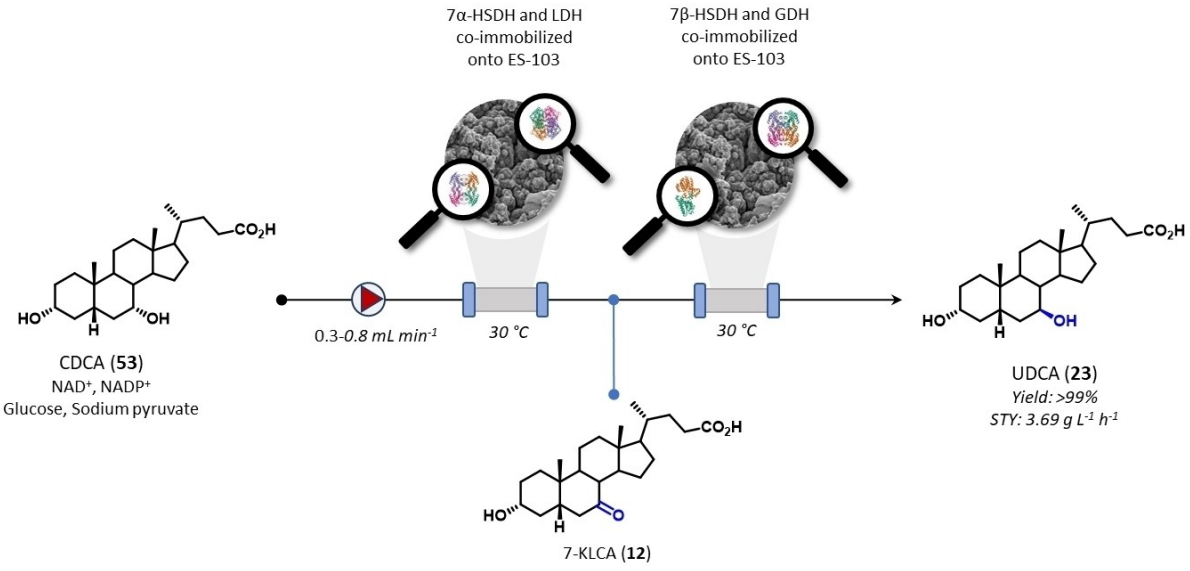
Scheme 16. Continuous enzymatic oxidation of cholesterol (**51**) to 4-cholesten-3-one (**52**) in microchannel reactors.

the reuse of the aqueous phase, maintaining 30% catalytic activity of cholesterol oxidase (Scheme 16).

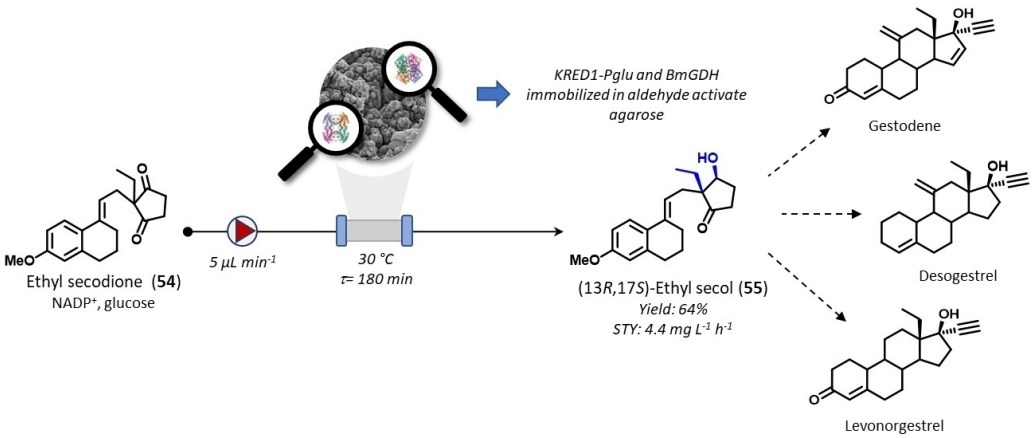
An efficient enzymatic cascade was applied for the conversion of CDCA (**53**) into UDCA (**23**) (Scheme 17).[53] The cascade reaction involved the oxidation of the C7-hydroxyl group using co-immobilized 7α-hydroxysteroid dehydrogenase (7α-HSDHEc) and a lactate dehydrogenase (LDH) for cofactor regeneration. The thus formed 7-KLCA (**12**) was then reduced by co-immobilised 7β-hydroxysteroid dehydrogenase (7β-HSDHRt–M1) in the presence of a glucose dehydrogenase (GDH) as cofactor-recycling enzyme. Among all the different immobilization methods investigated, cross-linking with available epoxy resin ES-103 proved to be the most effective approach to obtain good activity and stability. In particular, comparison between free and immobilized enzymes showed higher activity for the former and higher thermal stability for the latter. Moreover, when performed under conventional batch conditions, the biocatalyst activity decreased after five cycles due to mechanical stress and physical loss during the enzyme recovery procedures. The stock solution of the substrate CDCA (**53**) (10 mM) in a buffer containing 0.5 mM of NAD^+, 0.5 mM of $NADP^+$, 30 mM of sodium pyruvate and 30 mM of glucose, was pumped at the appropriate flow rate (ranging from 0.3 to 0.8 mL min^{-1}) using a peristaltic pump through two separate packed bed reactors connected in series. Optimization of reaction time, immobilization pH and biocatalyst loading maximized the activity recovery and the specific loading for both enzymes. Under these conditions, UDCA (**23**) was produced in 99% yield and a space-time yield of 3.69 g L^{-1} h^{-1} (Scheme 17).

In 2017, Serra, Tamborini and coworkers described the stereoselective reduction of ketones using a two-enzymes system consisting of immobilised ketoreductase (KRED1-Pglu) and glucose dehydrogenase (BmGDH) (Scheme 18).[54] Aldehyde-activated agarose was preferred over epoxy-activated support for immobilization since the latter resulted in a poorly active biocatalyst. The addition of 20% glycerol ensured improved enzymes stability over the immobilization reaction conditions (Scheme 18). Ethyl secodione (**54**), a key intermediate for the synthesis of several hormonal contraceptives, was included in the substrate scope. The solution of **54** (3.0 mM) in a Tris-HCl buffer (50 mM, pH 8) containing $NADP^+$, glucose (0.1 mM) and DMSO (20%, v/v) as co-solvent was pumped through an Omnifit glass column (V = 0.90 mL) pre-packed with mixed KRED1-Pglu and BmGDH (540 mg each, 1/25 U ratio) at a proper flow rate (τ = 180 min) (Scheme 18). The conversion to (13*R*,17*S*)-ethyl secol (55) was 64% during day 1 (space-time yield = 4.4 mg L^{-1} h^{-1}) (Scheme 18), and slight decreased to 62–63% after 15 days, thus proving the operational stability of continuous transformation compared to the batch reaction, for which the catalytic activity was completely lost after 4 cycles of reuse.

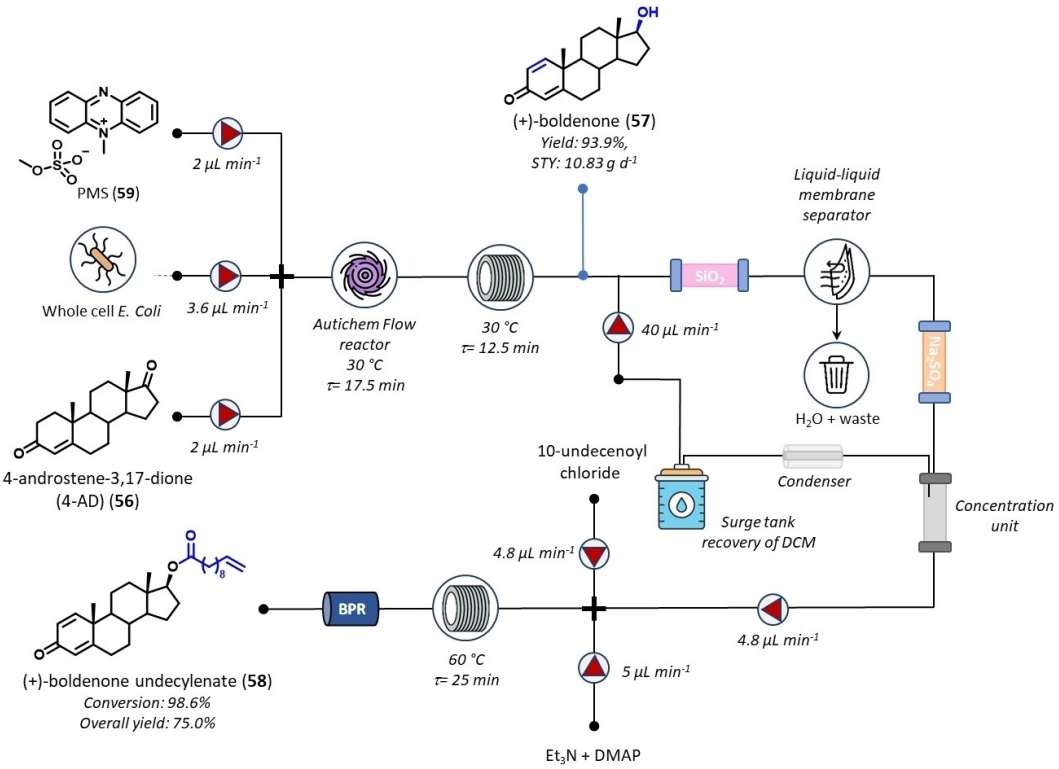
A very recent chemoenzymatic approach was reported by Chen and coworkers for the synthesis of (+)-boldenone (**57**) and the prodrug (+)-boldenone undecylenate (**58**) (Scheme 19).[55] The cascade reaction involved the Δ^1-dehydrogenation of 4-androstene-3,17-dione (4-AD, **56**), catalyzed by engineered 3-ketosteroid-Δ^1-dehydrogenase (Δ1KstD) ReM2, and the subsequent regio- and stereo-selective reduction of the C17-keto group promoted by a NADH dependent 17β-carbonylreductase. Upon optimization, an effec-



Scheme 17. Continuous flow synthesis of ursodeoxycholic acid (**23**) by a biocatalytic cascades using co-immobilized enzymes.



Scheme 18. Continuous flow stereoselective reduction of ethyl secodione (**54**) using co-immobilized enzymes.



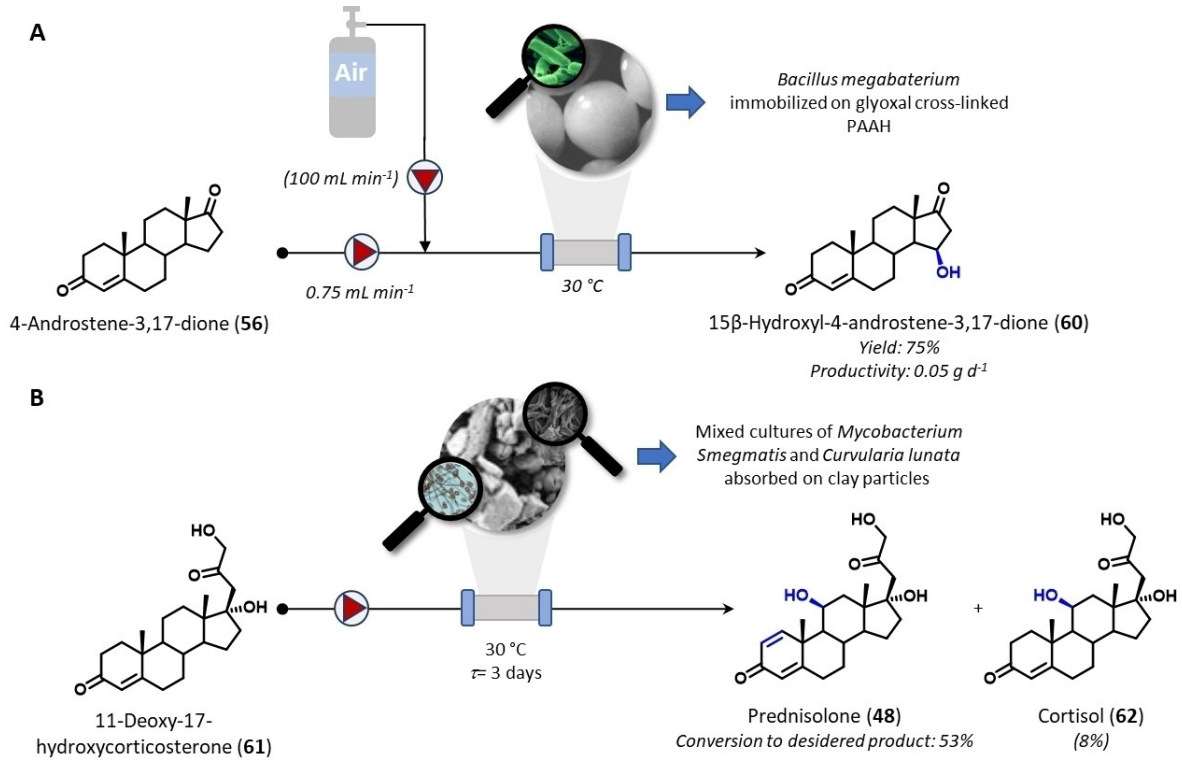
Scheme 19. Continuous flow single-cell asymmetric synthesis of (+)-boldenone (**57**) and its prodrug (+)-boldenone undecylenate (**58**).

tive single *E. Coli* cell bi-enzymatic strategy was achieved and tested initially in batch mode and then under continuous flow conditions. Three solutions were separately loaded into the reactor: a) an aqueous solution of phenazine methosulfate (PMS, 2 mM, 2 µL min^{-1}) that acts as the electron acceptor, b) a solution containing the whole cells (100 mg mL^{-1}) in phosphate buffer (50 mM, pH 7.5, 3.6 µL min^{-1}), and c) a solution of 4-AD (**56**) (20 mM, 2 µL min^{-1}) in CH_2Cl_2/*i*PrOH. The use of the binary organic solvent system proved to be pivotal since CH_2Cl_2 significantly increased the solubility of the steroid while *i*PrOH acts both as co-solvent and co-substrate for NADH regeneration. To avoid clogging issues due to limited solubility of the steroidal substrate in the aqueous media, an Autichem flow reactor was employed and connected to a PTFE coil reactor (Scheme 19). Under these conditions, after a total residence time of 30 min, (+)-boldenone (**57**) was produced in 94% isolated yield (>99% optical purity), in a space time yield of

$10.83\ g\,L^{-1}h^{-1}$ (*vs* $1.09\ g\,L^{-1}h^{-1}$ of the batch mode). The aforementioned set-up was applied to the continuous flow synthesis of (+)-boldenone undecylenate (**58**) (Scheme 19). At the end of the enzymatic cascade, the biphasic mixture leaving the reactor was submitted to in-line silica filtration, separation and concentration. The stream was then pumped at 4.8 μL min^{-1}, mixed in a four-ways connector with a solution of 10-undecenoyl chloride (1.2 equiv.) at 5 μL min^{-1} and combined with a solution of Et_3N (1.5 equiv.) and DMAP (0.2 equiv.) flowed at 5 μL min^{-1}. The reaction mixture entered a second PTFE coil reactor (V = 2.4 mL, τ = 25 min) heated at 60 °C where the esterification at C17 position took place. As a result, (+)-boldenone undecylenate (**58**) was obtained in ~99% conversion and 75% overall yield.

7.2. Biotransformations with Monooxygenases

As mentioned, one of the most important biotransformations at the steroidal core concerns the hydroxylation reaction that is performed by enzymes belonging to the monooxygenase sub-class. One of the earliest examples under flow conditions dates back to 1995, when Freeman and colleagues reported the development of the selective 15β-hydroxylation of 3-oxo-Δ^4-steroids catalyzed by immobilized *Bacillus megaterium* whole cells (Scheme 20A).[56] *B. megaterium* strain (ATCC 13368) is endowed with a cytochrome P450 monoxygenase activity that enables the stereo- and regio-specific hydroxylation at C15β-position of the steroidal body. Interestingly, this ferredoxin reductase-ferredoxin-dependent monoxygenase showed the maximum performance at the stationary phase of the cell growth curve, while it was not affected by the addition of CYP-450 inducers. The enzymatic activity was successfully stabilized by simulating stationary-phase conditions in a continuously operating immobilized fluid bed reactor. In this context, preliminary investigation with *B. megaterium* cells immobilized on glyoxal cross-linked PAAH gave a progressive decrease of catalytic activity under repeated batchwise operation (50% activity loss within 9 h), due to fluctuations in the environmental conditions upon repetitive washing and storage cycles. Also in this case, the adoption of flow technology was strategic. To this aim, a fluidized-bed reactor (V = 150 mL) thermostated at 30 °C by means of a water jacket was loaded with immobilized cells under sterile conditions. The stock solution of 4-androstene-3,17-dione (**56**) (0.17 mM) in tryptic soy broth buffer was delivered at 0.75 mL min^{-1} by aeration ensuring both mixing and slight pressurization. In proximity of the reactor outlet, a semi-permeable metal screen was placed to retain the immobilized cells while allowing the passage of freely suspended cells. Under these conditions, more than

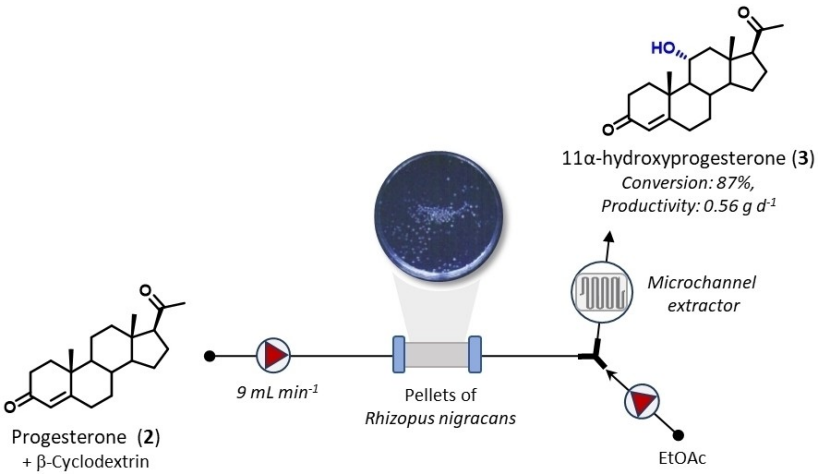


Scheme 20. Development of fluidized-bed reactors for (A) the stereoselective 15β-hydroxylation of 4-androstene-3,17-dione (**56**) and (B) conversion of Reichstein's substance S (**61**) into prednisolone (**48**).

50 h operational stability was achieved allowing the preparation of 15β-hydroxy-4-androstene-3,17dione (**60**) in 75% yield and productivity of 0.05 g d^{-1} (Scheme 20A).

The conversion of 11-deoxy-17-hydroxycorticosterone (Reichstein's substance S, **61**) into prednisolone (**48**) was performed by immobilized cultures of *Mycobacterium smegmatis* and *Curvularia lunata* (Scheme 20B).[57] Different immobilization techniques were investigated, including the entrapment on calcium alginate, K-carrageenan, agar-alginate and agar, as well as the adsorption on clay particles. As a result, the entrapment in calcium alginate gel ensured optimal microbial multiplication while the gel hydrophobicity improved both substrate uptake and conversion yield (82%). However, despite the slightly lower conversion efficiency (77%), clay particles were preferred as support being endowed with higher catalytic activity upon reuse. Thus, for the continuous flow transformation, a glass fluidized-bed bioreactor (V = 300 mL) was packed with clay particles, loaded with active fungal and bacterial cells and thermostated at 30 °C. The stock solution of 11-deoxy-17-hydroxycorticosterone (**61**) (10% in EtOH) diluted with the medium was fed by a peristaltic pump (τ = 3 d). Also in this case, the flow approach resulted superior to the batch modality in terms of conversions (61% *vs* 25%) and chemoselectivity (Scheme 20B).

Another example of continuous flow hydroxylation of steroidal compounds was reported by Žnidaršič-Plazl and co-workers, who developed pellets of *Rhizopus nigracans* as naturally immobilized biomass to perform hydroxylation of progesterone (**2**) to yield 11α-hydroxyprogesterone (**3**) in a lab-scale stirred tank bioreactor (Scheme 21).[58] In this regard, the addition of β-cyclodextrin (CD) in the medium was instrumental to improve the low aqueous solubility of the steroidal substrate. Complexation of **2** with β-CD increased the steroidal concentration in the medium without affecting the biotransformation rate, and attenuated the inhibitory effect exerted by free steroid molecules in the medium on the microbial strain. Batch and continuous experiments were performed with pellets of *R. nigracans* of different average diameters ranging from 0.8 to 7.5 mm. As expected, the best results were obtained using smaller pellets, thanks to their higher surface area and better transport properties. Authors also applied a previously developed mathematical model to describe the enzyme kinetics and to simulate both batch and continuous operation. In particular, the continuous process was performed in a laboratory-scale stirred tank bioreactor provided with a continuous outflow and a perforated glass filter at the outlet pipe to keep the pellets inside the reactor chamber. The reactor was continuously fed with a solution of progesterone (**2**) (0.16 mM) in the buffer containing β-CD (250 mg L^{-1}) at 9 mL min^{-1} of flow rate, while the reactor volume was kept constant by setting a continuous outflow of the reaction product at the same flow rate (Scheme 21). A further improvement was lately reported by the same authors that showed the advantages of integrating a pressure-driven flow microchannel system for product extraction.[59] The extraction of **3** from the aqueous medium was performed in a microreactor provided with y-shaped inflow and outflow and 26 bends, using EtOAc as the organic solvent. The flow rates of the aqueous and organic solvents were adjusted to establish the interphase at the middle of the channel and avoiding slug-flow formation, which would prevent phase separation. A three-dimensional mathematical model was also proposed to analyze and predict the extraction performance considering crucial parameters as the convection and diffusion terms, and the total flow rate.



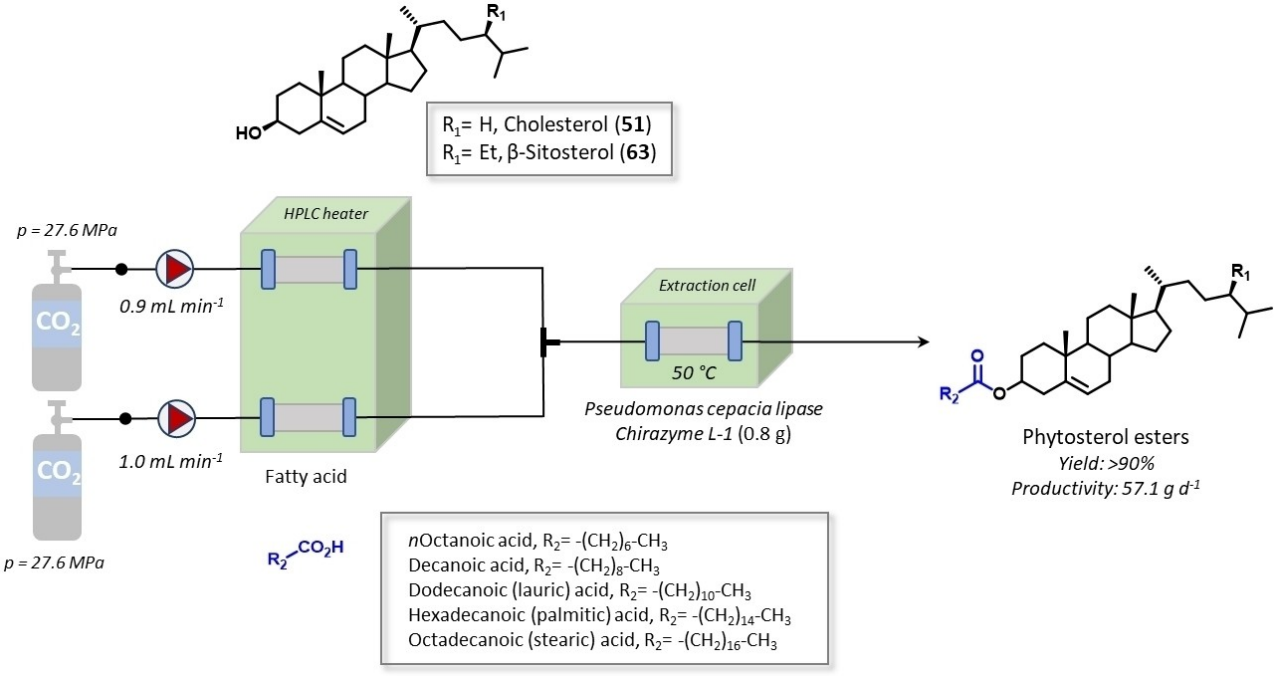
Scheme 21. Continuous flow 11α-hydroxylation of progesterone (**2**) by *Rhizopus nigricans* pellets packed into a stirred tank bioreactor.

Worth of note, the comparison between experimental data and model calculations were in good agreement, demonstrating a higher extraction yield at the steady-state conditions with respect to conventional gravity extraction. The desired product **3** was obtained with 87% conversion yield (productivity = 0.56 g d^{-1}) (Scheme 21).

7.3. Biotransformations with Hydrolases

One of the most investigated biotransformations under flow conditions is the lipase-catalyzed esterification of phytosterols such as cholesterol (**51**) and β-sitosterol (**63**). To the best of our knowledge, the first example of this approach was reported by Neese and co-workers in 2001,[60] wherein supercritical carbon dioxide was used as a green solvent (Scheme 22). CO_2 is indeed ideal for food industry application since no solvent residue is left in the product after the process. After preliminary optimization of the reaction parameters (type of commercial enzyme, temperature, pressure, static time) under batch or semi-continuous flow conditions, the authors switched to a flow set-up. The sterol and the fatty acid (1.4 to 2.3 equiv. depending on the acid) were separately loaded onto a reservoir placed in an HPLC heater. The two reactants were fluxed into the reactor through two syringe pumps (at a flow rate of 0.9 and 1.0 mL min^{-1}, respectively) filled with CO_2 at a pressure of 27.6 MPa. The reactor was pre-loaded with the selected enzyme (0.8 g L^{-1}, Chirazyme) and both the reactor and the heater were kept at 50 °C. Under these optimized conditions, phytosterol ester yields of over 90% could be obtained, with a remarkable productivity (57.1 g d^{-1}) (Scheme 22). Another example of lipase-catalyzed esterification of phytosterols was recently reported by Sengupta and colleagues using a packed bed reactor loaded with commercial immobilized *Thermomyces lanuginosus* lipase.[61] The method was applied to different fatty acids and lead to faster transformations and higher conversion yields.

An interesting alternative to the packed bed reactor consists in the use of cellulose monolith reactor.[62] Monolith structure presents a double-hole pattern consisting of macropores for liquid flow and nanoscale mesopores that are suitable for enzyme entrapment. This configuration has the advantages of avoiding for pre-packing, improving mass transfer of reagents due to the organized structure and controlling over contact time and flow rate, while simultaneously ensuring accessibility of the substrate to the lipase's catalytic site. β-Sitosterol (**63**) was selected as model substrate and the reaction parameters such as temperature, sterol/oleic acid ratio, and flow rate were optimized. Under the optimized conditions, a pre-heated and stirred solution of **63** (100 mM) and oleic acid (6 equiv.) in *i*-octane was pumped by a peristaltic pump (0.35 mL min^{-1}) through the AYS@CA-MN monolith (5% of the total substrate weight) reactor, heated at 55 °C. Under these conditions, the catalytic activity remained unchanged for 200 h demonstrating the operational stability of the system and resulting in



Scheme 22. Continuous flow C3-esterification of phytosterols by supercritical carbon dioxide.

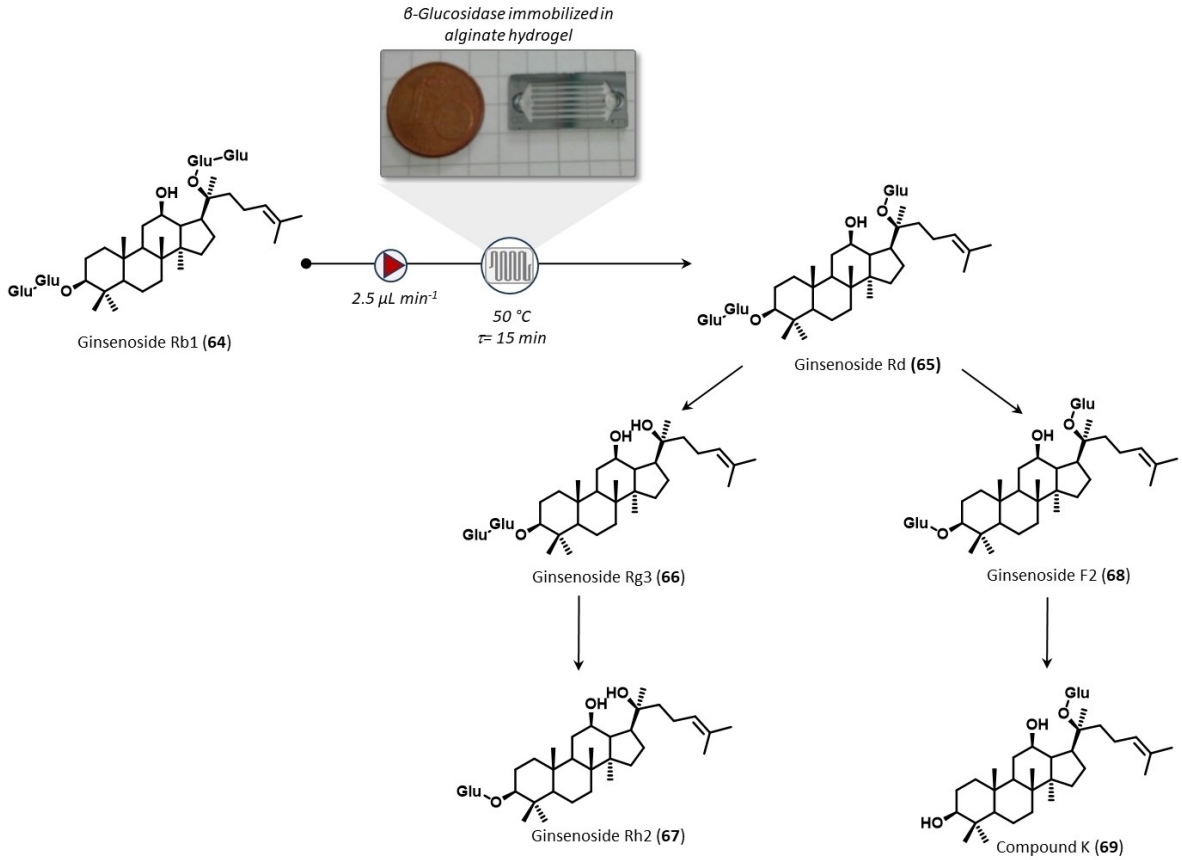
2.11 g h^{-1} of productivity. On the contrary, under batch conditions a complete loss of activity was observed after 4 cycles. Moreover, the comparison between continuous flow and batch approaches using both immobilized and free enzyme showed improved values for both V_{max} and V_{max}/K_M (59.2 and 66.4-fold higher, respectively). Different acyl donors (fatty acids and triglycerides) were also tested with conversion above 90%.

Later, the same group conducted the same reaction using lipase immobilized on hollow mesoporous silica spheres.[63] This support ensures high mechanical and thermal stability, biocompatibility, as well as provides good stabilization of the lipase. Under the optimized conditions, the operational reactor activity was stable for 10 days before showing a slow decrease over the time. However, the reactor performance was remarkable compared to the batch conditions, where only 20.1% conversion was observed after 20 cycles. Using this flow bioconversion, it was possible to sustain the reaction for 30 days with conversion above 95% and a productivity of 2.38 g h^{-1}, the highest value for PEs biosynthesis.

One of the most recent examples of continuous biotransformations of steroidal substrates was conducted for the hydrolysis of ginsenoside Rb1 (**64**), a bioactive compound from panax ginseng (Scheme 23).[64] The biocatalytic hydrolysis of these glucose-containing saponine is intriguing since it gives access to more active and unnatural occurring deglycosilated metabolites, which are difficult to obtain through conventional chemical hydrolysis. The enzyme β-glucosidase was immobilized on alginate hydrogel and the so-obtained material was injected onto a glass-silicon-glass micro reactor. The substrate solution was pumped with a syringe pump at a flow rate of 0.25 µL min^{-1}. Compared to the batch conditions, the microreactor led to a decrease in the conversion of ginsenoide Rb1 (**64**) (probably due to the reduction of the catalytic activity caused by the immobilization in the microreactor) that was compensated by a 13-fold higher amount of derivative F2 (**68**) due to the higher residence time inside the branched alginate-gel network.

8. Conclusions

Over the last decade, the steroidal market has experienced a growing increase that was further boosted due to SARS-CoV2 pandemic (4–5% annual growth).



Scheme 23. Continuous flow hydrolysis of ginsenoside Rb1 (**64**) using β-glucosidase-based microreactor technology.

From an industrial perspective, there is the unremitted need to develop new approaches to sustain market demand and to satisfy the modern requirements of sustainable production of steroidal APIs. In this scenario, continuous flow chemistry represents a powerful technology to streamline large-scale processes of existing steroidal drugs as well as to accelerate the synthesis of new steroid-based chemical entities. Flow chemistry can be integrated into multistep synthesis in combination with batch steps to facilitate the optimization, conduction and scale-up of critical steps. However, the translation of steroid chemistry into flow systems is still limited mainly due to the poor solubility of steroids in most of the organic solvents and their unpredictable reactivity in response to diverse reaction conditions. While the use of flow chemistry technologies for steroid synthesis is still in its early stage, benefits and opportunities can already be recognized. The integration with auxiliary technologies such as photochemistry and biocatalysis has been demonstrated effectively in respects of enhanced reaction yield and selectivity, excellent reproducibility and easy scale-up, and safe proceeding hazardous process. The rapid synthesis optimization, which has been enabled in continuous flow devices using design algorithms and automation, is essential to furnish sufficient quantity for structure-activity relationships during drug discovery and to evaluate compound efficacy in clinical settings. We strongly believe that the pace of change in steroid synthesis needs to move well beyond the simple target to address wider challenges and opportunities. This can be coped by restructuring education and research in chemical technology and innovation, as well as by improving the quality of collaborations between academia and industry.

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