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Biocatalytic Combinatorial Synthesis

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Abstract—Combinatorial biocatalysis, based on a principle of the combinatorial use of biosynthetic steps rather than the combinatorial use of reagents, offers a complementary approach to combinatorial chemistry, which, used individually or in connection with synthetic organic transformations, provides access to analogues not readily accessible by chemical synthetic means alone. The issues and strategies particular to this approach are discussed. Examples are given demonstrating these principles as well as the unique advantages of achieving chemo-, regio- and stereoselectivity under mild reaction conditions that biocatalytic methods offer.

Introduction

Combinatorial synthesis has found wide utility in a number of industries where the screening of arrays of compounds for desired properties has proven to be an efficient way to discover new potential products[1,2] or improve the properties of existing materials.[3,4] Most commonly, combinatorial synthetic methodologies operate from a model of total synthesis from sets of precursors systematically combined to produce groups of product molecules.[5–7] Particularly powerful examples of this approach are a number of multicomponent condensation reactions that have been previously described.[8]

An alternative model for combinatorial synthesis is one based on derivatization of existing molecules rather than their total synthesis. The difference inherent in this model is that arrays of reaction sequences are performed on a given molecule rather than a given chemistry performed on an array of precursor molecules (Fig. 1). This paper will examine the issues involved with this approach, with an emphasis on biocatalytic and chemoenzymatic reactions, give some examples of its implementation, and describe how it complements and can be combined with the total synthesis strategy to create a more powerful, integrated approach to combinatorial chemistry.

The derivatization approach represents much of the chemistry that occurs in biological systems where precursors or xenobiotics are modified by the action of biocatalysts to create physiologically active materials or their intermediates or for modification to deactivate or prepare for elimination from the body. Semisynthetic chemical approaches to natural product derivatives have proven useful in a number of instances, but the approach runs into difficulties with labile molecules or ones with functional groups that require protection from the reagents needed. Biocatalytic derivatization offers a number of advantages over chemical synthesis when working on complex molecules and offers a general approach towards a synthetic derivatization strategy.[9,10]

The synthetic uses of biocatalysts have been reviewed extensively[11,12] and will not be covered here. The key advantages that biocatalysis offers over synthetic chemistry approaches are the chemo-, regio- and enantioselectivity of enzyme catalysed reactions and the ability to work under mild reaction conditions. Even in cases where a simple synthetic chemical procedure exists for a given biotransformation (e.g. acylation) the chemo- and regioselectivity of the biocatalytic approach may eliminate several extra synthetic steps resulting from the need for protection/deprotection strategies in the chemical route.

At the risk of oversimplification, biocatalysts can be divided into two general categories: catalysts with narrower specificity, such as many of those important for the formation of biomolecules, and catalysts with broad specificity, such as many of those important for the modification or degradation of biomolecules or xenobiotics. The former can catalyze an impressive array of reaction types, including carbon–carbon bond forming

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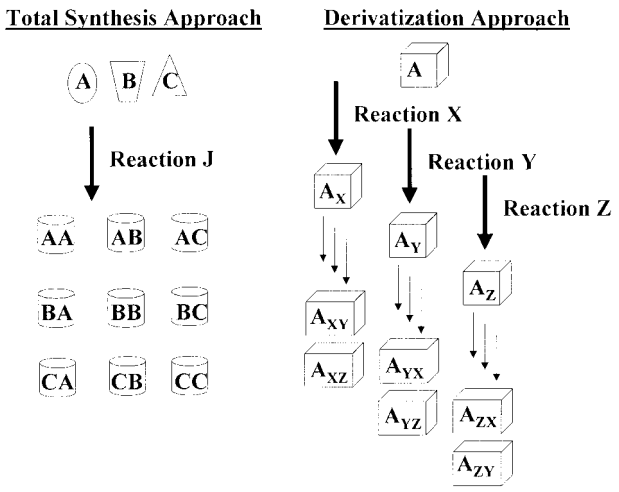

Figure 1. Comparison of combinatorial synthetic strategies.

reactions, but they are usually fairly specific for the types of molecules that they can modify. There are efforts underway to harness the synthetic utility of these biosynthetic catalysts through pathway engineering strategies.[13,14] The latter type of biocatalyst has naturally evolved to recognize and modify a broader substrate range. These less specific biocatalysts have wider utility for the synthetic modification of 'unnatural' substrates (Fig. 2).

The ability of biocatalysis to serve as a general synthetic methodology requires the ability to routinely transform a large number of unnatural substrates ranging from small molecules to complex natural products. To meet this requirement for a given transformation, a group of enzymes with non-overlapping specificity is usually needed to provide a reasonable expectation that a broad range of substrates can be effectively modified. Clearly there are molecules possessing functional groups that cannot be modified by any existing enzyme due to their steric or electronic nature; however, it is often not widely appreciated just how broadly many enzymes can be applied. One of the reasons for this is that for the majority of synthetic applications the most desirable biocatalysts are those that are highly specific for a particular process, and, therefore, the enzymes with relatively narrow substrate specificity become the focus of process development and biocatalyst screening. Once the enzyme with the desired specificity is found, the question of what else this or similar enzymes can do remains largely disregarded.

Still, the very nature of the specificity of enzymes creates some issues with regard to the iterative use of biocatalysts. One issue is that of orthogonality. Figure 3 illustrates this issue, The modification of a substrate by one enzyme, 'X', may preclude it from being a substrate for another enzyme, 'Y', while the modification provided by the other enzyme, 'Y', may not preclude it from being a substrate for the first enzyme, 'X'. The more tolerant the enzymes are of structural differences, the less is the issue of access to the dimodified analogues. Still, with at least two routes to each dimodified compound, the opportunity to make each possible combination is reasonably good. An example of this is the iterative enzymatic derivatization of bergenin (**1**) to produce 7-chloro-11-(α-galactosyl)bergenin (**3**) (Fig. 4). This product can be potentially synthesized via two different enzymatic routes: either chlorination followed by galactosylation or galactosylation followed by chlorination. In practice, the first route does not work, since 7-chlorobergenin (**2**) is not accepted by α-galactosidase as a substrate, but the second route, initially forming 11-(α-galactosyl)bergenin (**4**), gives the desired product (**3**).

Another approach utilized in combinatorial biocatalysis exploits the differences in the specificity of enzymes to direct the synthesis of sets of analogues. Figure 5 depicts an example of this application. In this case both enzyme 'X' and 'Y' catalyze the same reaction, but at different positions in the substrate. Enzyme 'X' specifically modifies one position and enzyme 'Y' modifies the same position as 'X' and another one. By using 'X' alone, one can specifically modify the one position. By using 'X' then 'Y', one can obtain specifically dimodified analogues of the substrate. By using 'Y' alone, one can obtain dimodified analogues as well, but if the reaction incorporates another reagent (e.g. an acylation reaction) then these analogues will be symmetrically substituted; whereas in the 'X' then 'Y' case dissimilar groups can be incorporated in a controlled fashion. Finally, the dimodified analogues from either the 'X' then 'Y' or the 'Y'

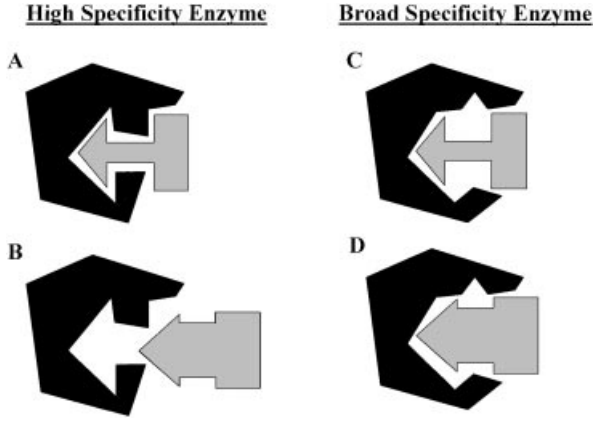

Figure 2. Comparison of high versus broad enzyme specificity.

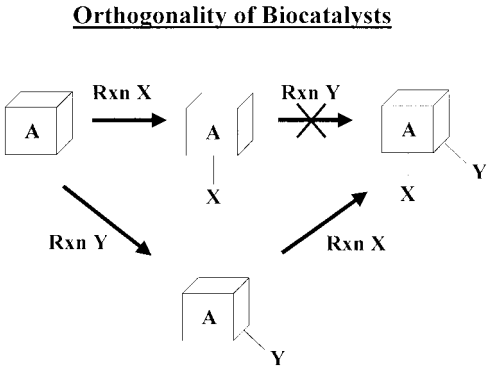

Figure 3. Orthogonality issues in iterative biocatalysis.

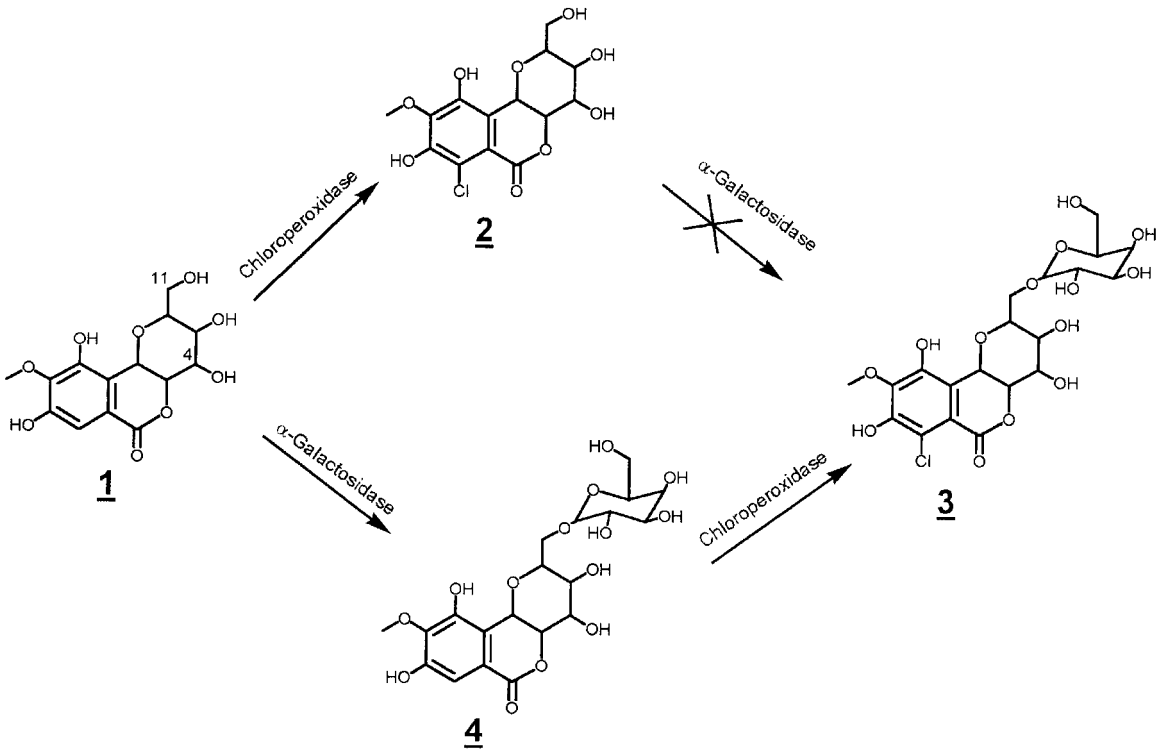

Figure 4. An example of the significance of reaction order on the production of analogues using iterative biocatalysis.

route alone can be exposed to enzyme 'X' in a reverse mode (depicted here as 'X': e.g. hydrolysis versus acylation) to give the monomodified analogues in the position where 'X' does not act. An example of this use of differing biocatalyst specificities is given in Figure 6.

Bergenin (**1**) can be selectively acylated at different positions using different enzymes.[15] For example, it was found that the mixture of lipases Chirazymes L-2 and L-9 (Boehringer), and lipases PS30 (Amano) and FAP-15 (Amano) catalyze regioselective acylation of the primary hydroxyl on the bergenin molecule giving **5**. On the other hand, subtilisin Carlsberg was found to produce a diacylated bergenin with substitutions at positions 4 and 11 (**6**, where $R^1 = R^2$). Moreover, when bergenin-11-butyrate (**5**, where $R^1 = n$-propyl) synthesized using the lipase catalyst was used as a substrate in subtilisin catalyzed acylation with vinyl acetate, only position 4 was acylated to give **6**, where $R^1 = n$-propyl and $R^1 = 2$-propene. Therefore, subtilisin can be used as a highly regioselective catalyst for 4-acylation of 11-substituted bergenin. Based on these observations, the following general three-step synthetic strategy was suggested for generating a library including all possible 4,11-mono- and diacylated bergenin derivatives as discrete compounds. In the first step, bergenin (**1**) is regioselectively acylated at the 11-position via lipase catalysis in dry acetonitrile. Yields were from 50 to 100% in 96 h depending on the individual acyl donor used in the reaction. When the reaction is complete, the immobilized biocatalyst is removed by filtration, and the 11-monoacylated bergenin derivative, **5**, is recovered by evaporating the acetonitrile. The clean product is then redissolved in a dry toluene–dimethylsulfoxide mixture, and added to immobilized subtilisin along with a second acyl donor. Because one of the positions on bergenin reactive to subtilisin-catalyzed acylation is already occupied (viz. position 11), the monoacylated product is selectively acylated at position 4, thus resulting in a homo- or hetero-4,11-diacylated bergenin derivative, **6**, depending on the acyl donor selected for the second step. The product can be recovered by filtering the solid enzyme, evaporating the solvent and extracting excess acyl donor. Finally, the regioselectivity of lipase for the 11-position can be used in the hydrolysis direction by replacing the reaction solvent with acetonitrile containing 2% (v/v) water, to give a quantitative yield of the selectively 4-monoacylated derivative, **7**. As in the previous steps, the product can be isolated by filtering the solid enzyme and evaporating the solvent. Using 96-well

Directed Analog Synthesis from Biocatalyst Specificity

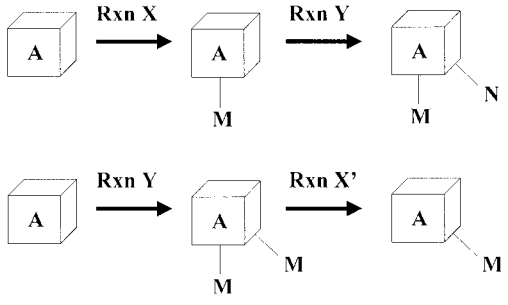

Figure 5. Catalyst specificity for the directed synthesis of specific analogues.

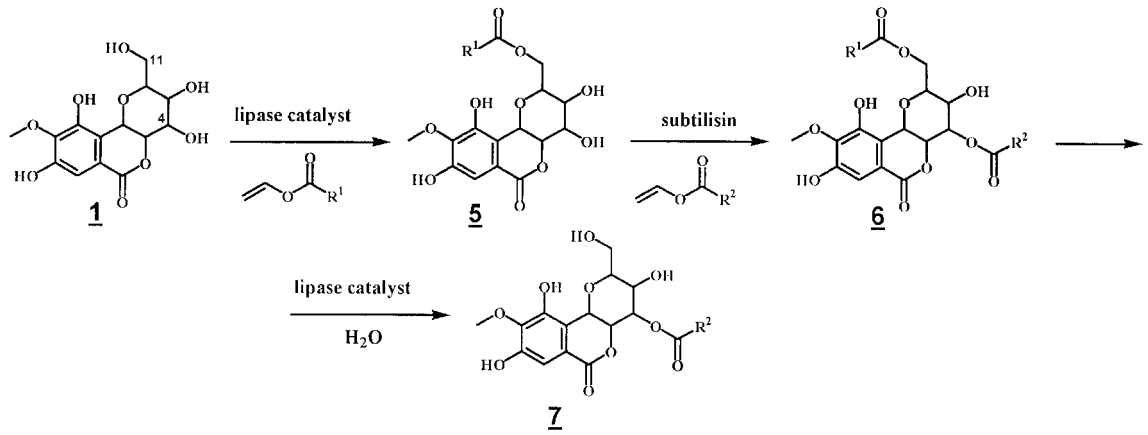

Figure 6. Use of differential specificity of enzymes catalyzing the same reaction to regiospecifically synthesize combinations of substitution.

polypropylene filter-bottom reactors, as described in the Experimental, an entire library of (n^2+2n) derivatives can be made in parallel by applying this scheme combinatorially using 'n' acyl donors. These types of approaches, taking advantage of the differing specificities of biocatalysts and the ability to use them in both 'forward' and 'reverse' directions, add great versatility and control to the iterative derivatization process.

One key barrier to using biocatalysis in a synthetic platform is the poor aqueous solubility of many substrates that one would wish to modify. While the natural environment of most enzymatic systems is aqueous, and their activity in organic solvents is generally dramatically decreased, methods have been described that allow for the synthetic utility of enzymes in organic solvents, often leading to reactions not possible in aqueous environments.[16–21]

Finally, in order to use biocatalysis in a general way for syntheses, there are operational barriers one must overcome. In order to efficiently explore the complete spectrum of biocatalytic reactions possible on a given substrate using a battery of enzymatic and microbial systems, one must have an efficient means of running and analysing each of the possible catalytic systems for its ability to modify the substrate. Our approach to this problem is through the pre-optimization of each individual catalytic system to a common platform that can be

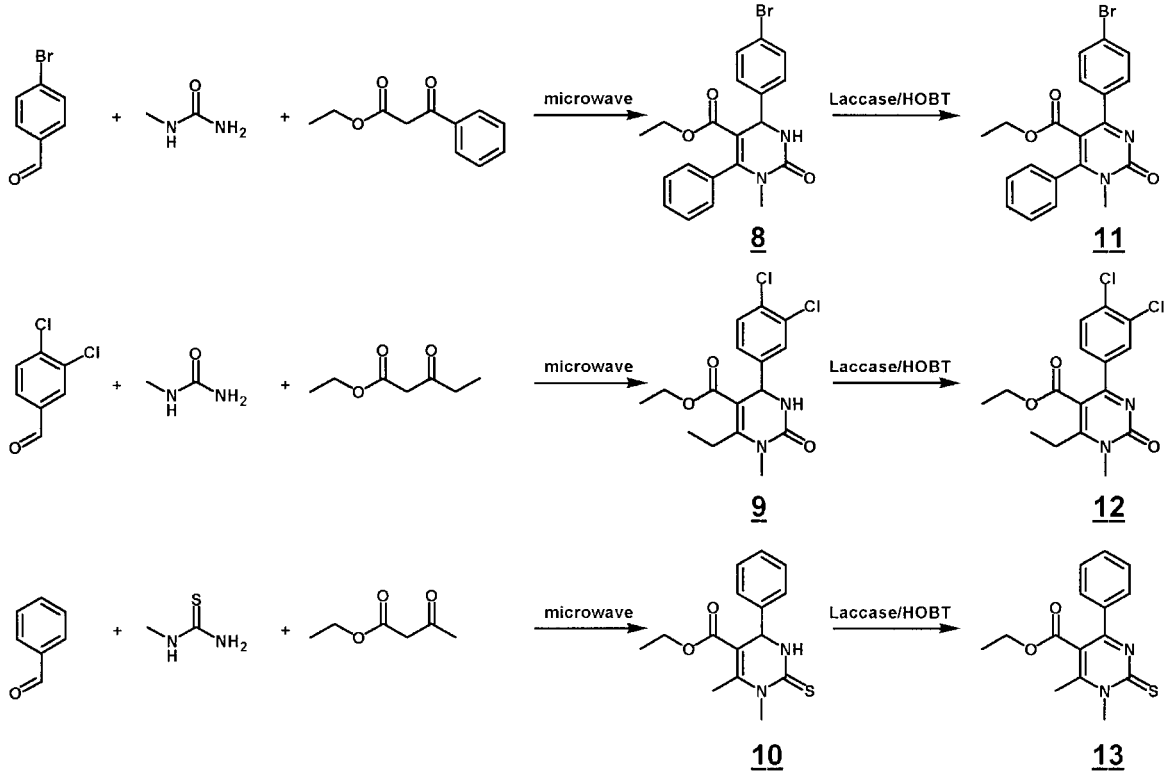

Figure 7. A Biginelli/laccase oxidation chemoenzymatic synthesis.

used for both screening and synthesis.[9,10] The common platform chosen is the familiar 96-well format used in most high throughput screening operations. The advantage of this system is the readily available equipment designed in the 96-well format for automating a wide variety of handling processes and its direct interface with the screening process. As automation and robotics have been important for combinatorial chemistry and parallel synthetic organic chemistry,[22,23] similar benefits can be seen with combinatorial biocatalysis. One operational advantage in the use of many biocatalysts is that enzymes that might be quite different and catalyze different reactions often operate optimally within a similar temperature range. This allows for the running of different types of reactions in the same 96-well plate in adjacent wells allowing for flexibility in strategies used in automated syntheses.

As powerful as biocatalysis is on its own, there are still areas of synthetic chemistry that biocatalysis does not address. One such area is the general creation of ring systems, particularly heterocyclic core structures. Considerable work in the combinatorial chemistry area has focused on various means of creating large numbers of derivatives of diverse core structures, both in solution and on solid-phase. Another method that has yet to see extensive use in the combinatorial area is microwave assisted organic synthesis (MAOS).[24–26] The method uses microwave energy to induce the reaction of reagents absorbed onto a solid material in a solvent-free environment. This method proves to be a convenient means of interfacing organic synthesis with a biocatalytic synthetic platform. MAOS can be performed in deep 96-well plates, which can then be processed using the same equipment that is used for the biocatalytic steps.[27] Thus, the addition of chemical steps coupled to biocatalytic steps can lead to unique 'chemoenzymatic' products, that would not be easily achieved by chemical or biocatalytic methods alone.[28–32] Fig. 7 outlines such a chemoenzymatic procedure involving a microwave-assisted Biginelli synthesis followed by laccase-catalyzed oxidation.

Conclusion

Thus, combinatorial biocatalysis offers a complementary approach to combinatorial chemistry, which, used individually or in connection with synthetic organic transformations, provides access to analogues not readily accessible by chemical synthetic means alone. With access to a collection of biocatalysts with varying specificity, one can apply strategies to obtain analogues with specific patterns of substitution without the need to employ complex protection/deprotection strategies. The mild conditions typically employed make these biocatalytic approaches compatible with molecules representing a broad range of molecular size and complexity. By further adapting these biocatalytic systems to a common automated platform that interfaces directly with modern high throughput screening methods, one can perform the needed catalyst screening and reaction optimization steps in an efficient manner. This common platform can then be applied to any type of problem of lead discovery and lead optimization in a variety of industries (e.g. pharmaceutical, agrochemical, etc.) to provide novel analogues in a timely manner.

Experimental

Biocatalytic chlorination. The reaction mixture contained 16 mM bergenin (**1**) or 11-(α-galactosyl)bergenin (**4**) and 1.5 mg/mL chloroperoxidase from *Caldariomyces fumago* (Sigma) in 100 mL 0.1 M phosphate buffer, pH 2.8. The reaction was carried out at room temperature by slowly adding 0.1 mL of 0.36 M hydrogen peroxide solution in the same buffer over the period of 2 h. The reaction was then freeze-dried and the product was extracted from the dry residue with methanol. The product was purified by flash chromatography on a silica column using ethyl acetate: chloroform:methanol (5:4:2) as eluent. The yield was 50% for chlorination of bergenin (**2**) and 20% for chlorination of 11-(α-galactosyl) bergenin (**3**).

Biocatalytic α-galactosylation of 1. The reaction mixture contained 25 mM bergenin (**1**), 25 mM *p*-nitrophenyl α-galactopyranoside, and 3 units/mL α-galactosidase from coffee beans (Sigma) in 70 mL sodium phosphate buffer, pH 6.5, containing 25% acetonitrile. The reaction was carried out at room temperature for 3.5 h. The reaction was then stopped by boiling the reaction mixture for 15 min. The product was purified by flash chromatography on a silica column using isopropanol: ethyl acetate: methanol (6:12:1) as the eluent. The yield was 20% of 11-(α-galactosyl)bergenin (4).

Regioselective acylation of bergenin (1). All reactions were performed in 96-well (2 mL/well) glass-filled polypropylene filter plates (10 μm polypropylene filter, Polyfiltronics, Rockland, MA). A custom sealing clamp and septa were used to allow sampling and prevent evaporation of the organic solvent during the reaction.

For the first acylation step, 15 mg of the immobilized lipase mixture (equal parts of PS30, FAP-15, Chirazyme L-2, and Chirazyme L-9) were added to each well in the plate. Using a Cyberlab C-200 liquid handler (Brookfield, CT), 25 mM of bergenin (**1**) and 500 mM of the appropriate acyl donor in acetonitrile were added to the 96-well plate (final reaction volume 1 mL). The sealed 96-well plate reactor was then shaken (250 rpm) at 45°C. Periodically, samples were automatically withdrawn and analyzed by HPLC and/or high-throughput MS (flow injection at ca. 1 sample/min). After 96 h the first acylation reaction was completed (yields of 50–100% depending on the individual acyl donor), the enzyme was removed by filtration through the reactor bottom, the solvent removed under vacuum using a Savant SpeedVac Plus centrifugal evaporator with a microplate rotor, and the excess acyl donor removed by washing (5x) with hexane.

The second acylation step was performed in an identical fashion to the first step (96 h at 45°C). Subtilisin/95% KCl, 40 mg, was added to each well in the plate and toluene containing 5% (v/v) dimethyl sulfoxide was added for a total reaction volume of 1 mL; 2 mM of bergenin derivative, **5**, and 50 mM of the appropriate acyl donor were used for the second step to give 50–80% yields depending on the individual acyl donor used.

In the third hydrolysis step the same lipase mixture as in the first step above was used as a catalyst. The lipase mixture (50 mg) was added to 1 mL of 5–20 mM solution of 4,11-diacylated bergenin, (**6**), in MeCN containing 2% (v/v) water. The reaction mixture was incubated under shaking (250 rpm) at 45°C for 96 h. Upon completion of the reaction, the solid enzyme was removed by centrifugation and the products, **7**, were obtained in quantitative yield and recovered by evaporating the solvent in vacuum.

Microwave-assisted Biginelli synthesis. Aldehyde (0.075 mmol), 1,3-dicarbonyl compound (0.13 mmol), and urea or thiourea (0.05 mmol) were dissolved in 0.2 mL ethanol containing 2 drops of glacial acetic acid. The solution was mixed in a glass vial with 200 mg of montmorillonite K-10 clay and the solvent was removed under reduced pressure. The mixture was treated in a 1300 W domestic microwave oven for 20 min at 40% power. The product was extracted with 2 mL ethanol, and the solution was stirred overnight at room temperature with 100 mg 3-aminopropyl silicagel to remove the excess of starting reagents. The purified product was recovered by removing the solvent under reduced pressure. Isolated yields were 38% (**8**), 64% (**9**), and 43% (**10**).

Laccase-catalyzed oxidation. The reaction mixture was prepared by dissolving the product of the Biginelli reaction (2 mM) and 1-hydroxybenzotriazole (HOBT, 1 mM) in 0.5 mL benzene. The reaction was started by adding 90 μL solution of laccase from *Coriolus versicolor* (ASA Spezialenzyme, Salzgitter, Germany) in 50 mM phosphate buffer pH 6.5. The enzyme concentration in the final mixture was 7.2 syringaldazine units/mL. The biphasic reaction mixture was incubated at room temperature under shaking at 250 rpm for 3 days. The reaction mixture was then diluted with 0.6 mL of isopropanol-DMF mixture (2:1), the enzyme removed by centrifugation, and the product recovered by removing the solvent in vacuum. Analysis of the products by HPLC/MS showed the following conversions: 42% for **11**, 73% for **12**, and 34% for **13**.

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