Pergamon

Tetrahedron 54 (1998) 3971–3982

TETRAHEDRON

Regioselective Enzymatic Acylation as a Tool for Producing Solution-Phase Combinatorial Libraries

Vadim V. Mozhaev[≠], Cheryl L. Budde[¶], Joseph O. Rich[¶], Alexander Ya. Usyatinsky[¶], Peter C. Michels[¶], Yuri L. Khmelnitsky[¶]*, Douglas S. Clark[+], and Jonathan S. Dordick[≠]

[¶]EnzyMed, Inc., 2501 Crosspark Rd., Oakdale Research Park, Iowa City, IA 52242, USA

[+]Department of Chemical Engineering, University of California, Berkeley, CA 94720, USA

[≠]Department of Chemical and Biochemical Engineering and Center of Biocatalysis and Bioprocessing, University of Iowa, Iowa City, IA 52242, USA

Abstract: A simple combinatorial strategy for sequential regioselective enzymatic acylation of multifunctional lead compounds has been developed and demonstrated using a polyhydroxylated flavonoid, bergenin, as a model. The approach is based on the ability of different enzymes to regioselectively acylate different sites on a lead molecule without affecting other similar functional groups. In sharp contrast to enzymatic acylation, conventional chemical acylation methods showed almost complete lack of regioselectivity. The enzymatic strategy was applied successfully to produce a solution phase combinatorial library of 167 distinct selectively acylated derivatives of bergenin on a robotic workstation in a 96-well plate format. General applicability of the automated combinatorial biocatalysis strategy is discussed.

INTRODUCTION

Biologically active lead molecules, either from natural isolates, rational design, or libraries from combinatorial synthesis typically contain multiple substituents that are be chemically reactive. Because biological activity relies on the specific chemistry and arrangement of functional groups of a compound, the ability to derivatize some functionalities regioselectively, while leaving others free for alternative modifications and/or to interact with the biological target, can be important for drug development. Traditional chemical methods are generally not selective enough to allow derivatization of polyfunctional compounds without protection/deprotection schemes. The additional steps and purifications introduced to a library synthesis by these steps can be prohibitive, especially for solution phase combinatorial syntheses.

0040-4020/98/$19.00
PII: S0040-4020(98)00129-X

Enzymes are regioselective catalysts that have been used for the regioselective synthesis of pharmaceutically important compounds.[1-8] For instance, the regioselectivity of alcohol dehydrogenases allows the facile, selective reduction of dicarbonyl intermediates in the synthesis of the mycotoxin, zearalenone,[1] and the calcium channel blocker SQ31,765.[2] Likewise, oxygenases have been used extensively for the regioselective hydroxylation of many therapeutics, including important steroid derivatives,[3] and for the selective oxidation of one of two methyls to a carboxyl group in the synthesis of the antidiabetic agent, glipizide.[4]

Regioselective enzymatic acylations have also played an important role in the development of pharmaceutical compounds. For example, the HIV replication inhibitor, castanospermine, contains four chemically similar secondary hydroxyl groups. The chemical synthesis of selectively acylated derivatives, which are more potent than the parent molecule, requires a tedious, multi-step chemical procedure. In contrast, the bacterial protease subtilisin and lipase from *Chromobacterium viscosum* can acylate specific hydroxyls directly to produce the more active derivatives in a single step and high yield.[5,6] Regioselective acylations have also been used to produce the azole antifungal compound SCH 56592 for Phase II clinical studies,[7] and for the synthesis of a variety of acylated carbohydrates, nucleoside analogs, and steroids.[8]

The well-recognized regioselectivity of enzymes is ideal for solution phase combinatorial strategies with polyfunctional lead molecules. Moreover, enzyme and whole cell biocatalysts operate under uniform, mild reaction conditions; they therefore can be applied for a wide range of reaction chemistries with minimal side products and simple work-up before further reaction. Solution phase combinatorial strategies are particularly well suited for addressing the challenges of lead optimization. Solid phase syntheses would require development of a method for linking and cleaving the lead molecule and its derivatives to/from a solid support, thus limiting the locations and types of reactions that can be performed to generate a derivative library.

In the present paper, some of the unique advantages of regioselective combinatorial biocatalysis for solution phase synthesis of chemical libraries are illustrated through the preparation of a 167-compound library of derivatives of the polyfunctional natural flavonoid, bergenin. The synthetic scheme focuses on the use of enzymatic acylation to produce a derivative library of this flavonoid that could not be prepared using a chemical combinatorial scheme of similar simplicity. Thus, the regioselectivity inherent in combinatorial biocatalysis offers a powerful complement to chemical methods.

RESULTS AND DISCUSSION

Bergenin contains five hydroxyl groups potentially amenable to enzymatic acylation (Fig. 1). To identify biocatalysts capable of regioselectively acylating specific sites on bergenin, we tested over fifty commercially available lipases and proteases as transesterification catalysts in organic solvents using vinyl

butyrate as a model acyl donor. Automation of the enzyme screen in 96-well polypropylene plates and high throughput mass spectrometric analysis (1 sample/min) allowed the rapid identification of several lipases that acylated bergenin in anhydrous CH_3CN exclusively at the primary hydroxyl group (position 11 in Fig. 1) with nearly 100% conversion. The best enzymes included Chirazymes L-2 and L-9, and lipases PS30 and FAP-15. In subsequent experiments, a mixture of these lipases was used as a catalyst for regioselective acylation of the primary hydroxyl on the bergenin molecule. On the other hand, subtilisin Carlsberg suspended in a toluene-DMSO mixture (95 : 5 v/v) was found to produce a diacylated bergenin with substitutions at positions 4 and 11. Moreover, when bergenin-11-butyrate synthesized using the lipase catalyst was used as a substrate in subtilisin-catalyzed acylation with vinyl acetate, only position 4 was acylated.[9] Therefore, subtilisin can be used as a highly regioselective catalyst for 4-acylation of 11-substituted bergenin.

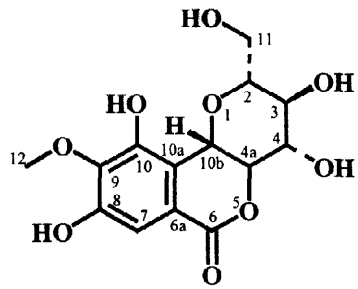

Fig. 1. Structure of bergenin

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 (Fig. 2). In the first step bergenin is regioselectively acylated at the 11-position via lipase catalysis in dry CH_3CN. When the reaction is complete, the immobilized biocatalyst is removed by filtration, and the 11-monoacylated bergenin derivative is recovered by evaporating the CH_3CN. The clean product is then redissolved in a dry toluene-DMSO 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, depending on the acyl donor selected for the second step. 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 MeCN containing 2 % (v/v) water, to yield a selectively 4-monoacylated derivative. As in the previous steps, the product can be isolated by filtering the solid enzyme and evaporating the solvent. Using 96-well polypropylene filter-bottom reactors, as described in the Experimental Section, an entire library of $N^2 + 2N$ derivatives can be made in parallel by applying this scheme combinatorially using N acyl donors.

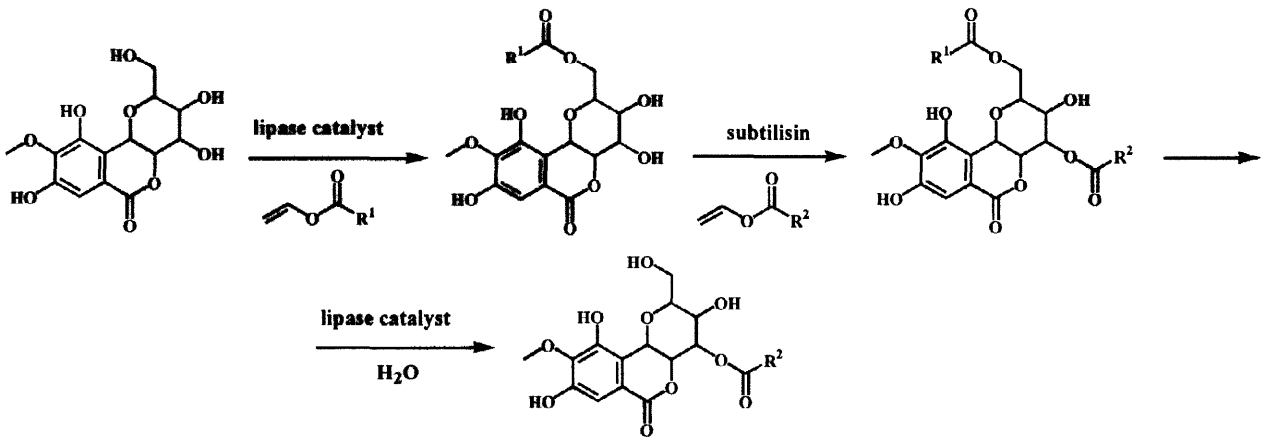

Fig. 2. Synthetic strategy for production of acylated bergenin derivatives by three-step regioselective enzymatic acylation/hydrolysis. The lipase catalyst was prepared by mixing equal weight parts of immobilized Chirazyme L-2, Chirazyme L-9, lipase PS30, and lipase FAP-15.

It should be noted that Fig. 2 describes a *general* strategy for generating the library of mono- and diacylated bergenin derivatives. Production of certain specific sublibraries can be accomplished by applying subsets of enzymatic acylations selected from this general scheme. For example, the sublibrary of 4,11-homodiacylated derivatives (R^1=R^2) can be produced in one step by acylating bergenin with a single acyl donor using subtilisin as a catalyst. Similarly, the sublibrary of 4-monoacylated derivatives can be obtained from corresponding 4,11-homodiacylated derivatives by lipase-catalyzed regioselective hydrolysis (*cf.* the third step in Fig. 2). The choice of actual synthetic strategy is dictated by experimental convenience and whether the reactions are set-up and purified using automated or manual procedures.

The successful application of this synthetic strategy is illustrated by Fig. 3, which shows the results of HPLC and mass spectral analysis of the products obtained in a sequential acylation of bergenin according to the scheme in Fig. 2. In this example, bergenin (chromatogram in Fig. 3A) was first converted to a monoacylated derivative in a lipase-catalyzed reaction with vinyl butyrate (chromatogram in Fig. 3B). The product obtained in nearly quantitative yield after 96 h was further reacted with vinyl 2-methylthiophenecarbonate in the presence of subtilisin to give a heterodiacylated bergenin (80% yield in 96 h, chromatogram in Fig. 3C). To illustrate the regioselective hydrolysis (third step in Fig. 2), 4-butyrylbergenin was generated from 4,11-dibutyrylbergenin by selective enzymatic hydrolysis of at the 11 position (85% yield in 96 h, chromatogram in Fig. 3D). Comparison of traces B and D in Fig. 3 clearly shows that the 11- and 4-monobutyrylated derivatives produced *via* lipase-catalyzed acylation of bergenin and selective hydrolysis of 4,11-diacylated bergenin (corresponding to the first and third steps in Fig. 2, respectively), have distinct retention times, but identical mass ions. Identities of all mono- and diacylated products were confirmed by HLPC/mass spectrometry.

The ability to selectively derivatize a single functional group on a multifunctional molecule is a unique feature of enzymatic catalysis that is typically very difficult to duplicate using conventional chemical methods. For example, when bergenin was chemically acylated with an equimolar amount of butyryl chloride, a mixture of several mono- and diacylated products was obtained, even at low conversions of bergenin (Fig. 3E). Similar results were obtained in acylation reactions of bergenin with benzoyl chloride or butyric acid/N,N'-dicyclohexylcarbodiimide. Clearly, in order to produce a library of selectively acylated bergenin derivatives using purely chemical methods, a complex synthetic scheme involving protection/deprotection steps would be needed.

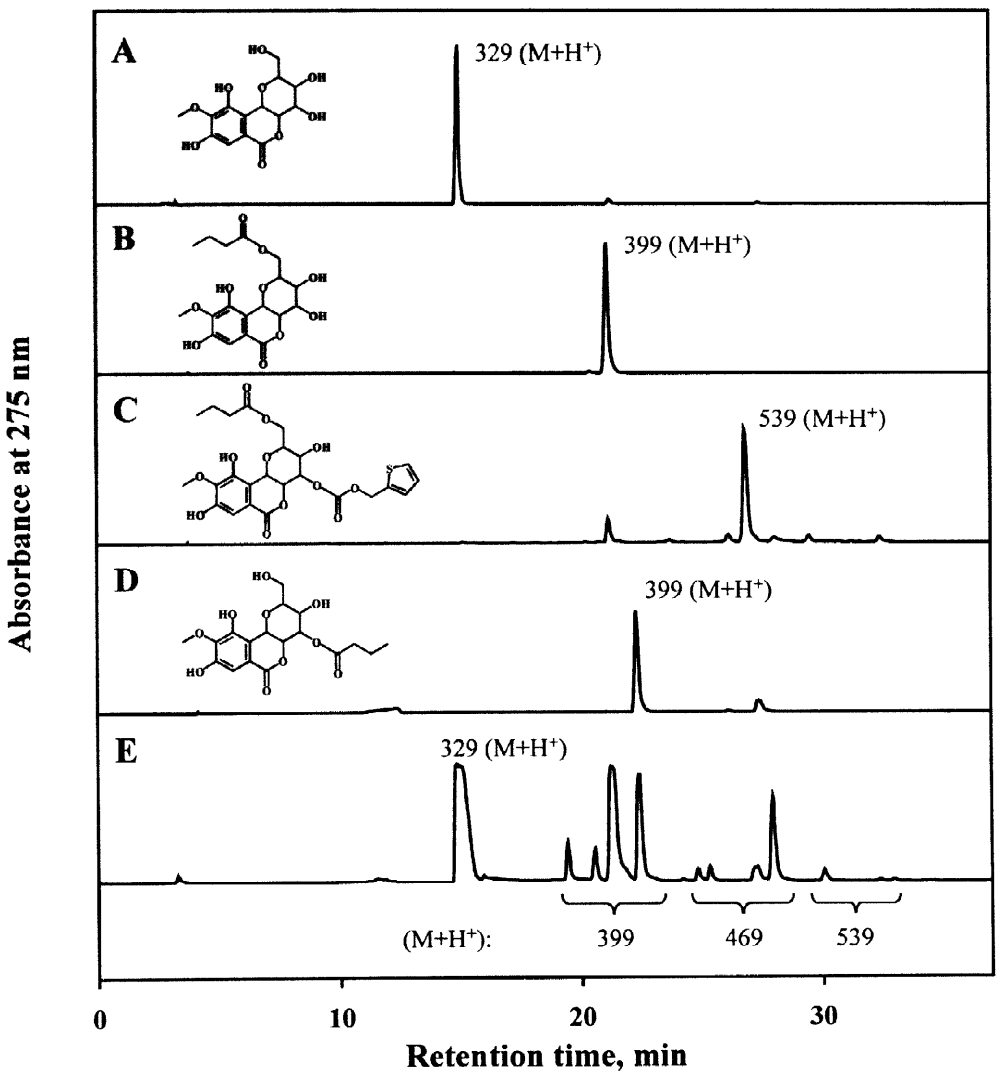

Fig. 3. Results of HPLC/MS analysis of the products of the three-step acylation/hydrolysis of bergenin. A. Starting bergenin. B. Product of acylation of bergenin with vinyl butyrate using the lipase catalyst. C. Product of acylation of the monoacylated butyrylbergenin with vinyl 2-methylthiophenecarbonate using subtilisin as a catalyst. D. Product of lipase-catalyzed hydrolysis of 4,11-dibutyrylbergenin. E. Product of chemical acylation of bergenin with butyryl chloride. Molecular weights 468 and 538 correspond to di- and triesters of bergenin and butyric acid, respectively.

The three-step enzymatic acylation/hydrolysis strategy was then applied to generate a library of mono- and diacylated bergenin derivatives. In this example, a set of twelve acyl donors of different types was used, including vinyl and trifluoroethyl esters and vinyl carbonates (Fig. 4). The acylations were performed combinatorially so that the twelve acyl donors were reacted with bergenin to produce all possible (24 monoacylated and 144 diacylated) derivatives as distinct/discrete compounds. All steps of the library synthesis, including reaction setup, sampling, and work-up, were performed using a robotic workstation as described in the Experimental Section. In order to achieve high conversions and increase the rate of enzymatic acylations, all acyl donors were used in 25-fold molar excess. Acyl donor remaining after completion of the first step was removed to prevent the formation of undesirable homodiacylated products in the second step. In most cases excessive acyl donors could be eliminated together with the reaction solvent by evaporation *in vacuo*. Less volatile donors were removed by extracting the dry residue obtained after solvent evaporation with hexane, which is a good solvent for the acyl donors but does not dissolve bergenin nor its derivatives.

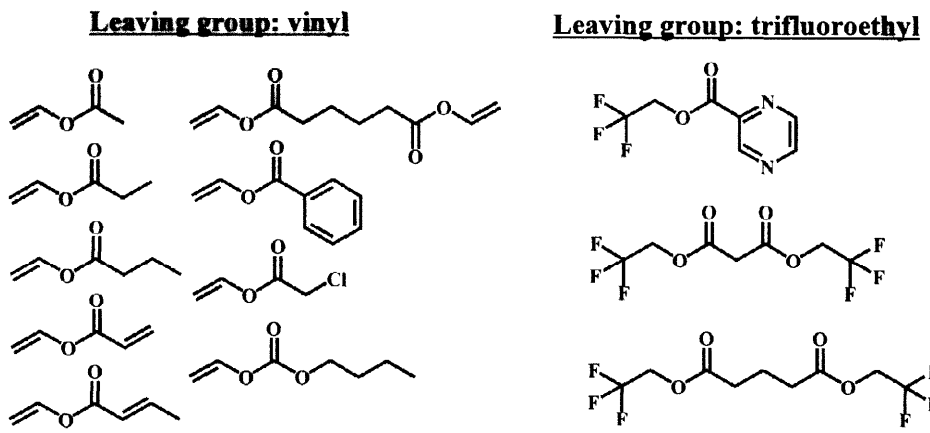

Fig. 4. Structures of acyl donors employed in the library synthesis. In the case of bifunctional acyl donors only one ester group of the acyl donor reacted, while the second was left intact.

Each of the bergenin derivatives synthesized using the three-step regioselective enzymatic acylation/hydrolysis was analyzed by HPLC/MS. In all cases except one the desired acylated products were found and showed the expected molecular mass in MS. The only unsuccessful derivative involved diacylation with two different bulky aromatic acyl donors (Fig. 4), which presumably impeded the second step acylation by creating steric hindrances at the active site of the enzyme. Based on HPLC data, overall yields of selectively acylated bergenin derivatives ranged from 60 to 90%.

In addition to the synthetic convenience afforded by regioselective enzymatic catalysis, the automation of library synthesis can be performed with inexpensive, readily available equipment and straightforward programming. A commercial robotic liquid handler, modified for operation with eight parallel septa-piercing

probes, was used to perform each step of library synthesis in commercially available 96-well plates. Reaction sampling and product recovery were simplified by the mild and non-corrosive solvents appropriate for biocatalysis, which allowed common septa materials to be used to seal the plate reactors. Biocatalysis also allows greater flexibility for the configuration of the synthetic process. In contrast to typical solid phase procedures for combinatorial synthesis, combinatorial biocatalysis can operate with either the substrate or immobilized catalyst in the solid phase. For instance, in the present work, immobilized lipases and subtilisin were used to selectively acylate bergenin, and the products were recovered in the solution phase by simple filtration from the catalysts through the bottom of the reactor. Since limited purification was required between the highly selective reactions, the necessary work-up could also be performed in 96-well plates on the robotic deck. The same simple equipment and procedures could be used for an iterative scheme involving a broad range of additional enzyme catalysts, which work under similar mild and uniform conditions for regiocontrolled synthesis.

The combinatorial biocatalysis approach described here is also unique in that it allows catalysts to be applied combinatorially to reaction development. In the present case, over fifty enzyme catalysts were screened in parallel as selective catalysts for the acylation of bergenin in a standard reaction, and 11 enzymes were identified as possible catalysts for the modification of bergenin. In this way, catalysts capable of acylating bergenin at different sites with various degrees of specificity were rapidly identified. Conceptually this process is very similar to high throughput screening for biological activity against enzyme targets, in which the reactions of a broad range of enzymes can be assayed using high density microplates and well established robotic liquid handlers. However, the development of automation for synthetic biocatalysis has not previously been described in the literature.

Conclusion

As has been extensively demonstrated in nature, combinatorial biocatalysis exploits the inherent, proven strengths of enzymatic synthesis to produce small organic compounds of therapeutic value.[10] The biocatalytic process demonstrated herein can likewise be expanded to include enzymes that catalyze a broad range of organic reactions, such as hydroxylation, glycosylation, phosphorylation, halogenation, oxidations, and reductions, while retaining the advantages of solution phase synthesis.[11,12] Thus, combinatorial biocatalysis is capable of generating large derivative libraries, starting from a diversity of lead structures, to identify compounds with improved therapeutic potential.

Some advantages of this approach demonstrated by the acylated bergenin library include the opportunity for regioselective control over the compound library, mild and uniform reaction conditions, minimal side products, and simple work-up before further reaction. Practically, these advantages allow single-step derivatizations on a broad variety of lead molecules or scaffolds, including fragile or

chemically/stereochemically complex leads. Numerous enzymatic chemistries can be performed using simple and inexpensive automated synthesis, work-up, and analytical tools, thus allowing efficient parallel synthesis of pure compounds. These advantages provide a desirable alternative to combinatorial chemical synthesis techniques for the discovery of new lead structures possessing biological activity, and especially for the optimization of existing leads to identify possible clinical candidates.

Many developing technologies offer promise to enhance the utility of combinatorial biocatalysis. Revolutionary approaches for enzyme discovery, via PCR-based approaches such as shotgun cloning or panning with degenerate primers, has already greatly expanded the diversity of available biocatalysts.[13] Moreover, recent advances in protein overexpression[14,15] and affinity tag purification[16,17] offer the prospect of obtaining significant quantities of pure enzymes for practical application. Novel mutagenesis and gene shuffling strategies allow the production of customized synthetic enzyme catalysts[18-21] and new enzyme activities.[22] In addition, the development of combinatorial biosynthesis allows biocatalytic pathways to be utilized for combinatorial synthesis of "unnatural natural products" *in vivo* by altering the organic precursors or enzyme catalysts of the natural product pathways.[23] The increasing impact of these approaches, along with the growing importance of combinatorial chemistry, will continue to expand the capabilities of combinatorial biocatalysis.

EXPERIMENTAL SECTION

Enzymes

Chirazymes L-2 (lipase from *Candida antarctica*) and L-9 (lipase from *Mucor miehei*) were from Boehringer Mannheim (Indianapolis, IN). The polymer-supported enzymes were used without further modification. Lipases PS30 (from *Pseudomonas* sp.) and FAP-15 (from *Rhizopus oryzae*) were from Amano (Lombard, IL). Subtilisin Carlsberg (protease from *Bacillus licheniformis*) was purchased from Sigma (St-Louis, MO).

Lipases PS30 and FAP-15 were immobilized on Accurel polypropylene beads (Akzo Nobel, Obernburg, Germany, 200-400 μm particle size) following published procedures.[24] Subtilisin Carlsberg was lyophilized in the presence of 95% KCl by using the procedure of Khmelnitsky *et al.*[25] to increase the catalytic activity of the enzyme in the organic solvent.

Solvents and Chemicals

All solvents were purchased from commercial suppliers and were of the highest purity available; they were stored over 3 Å molecular sieves (Linde). Analytical grade solvents for thin layer and flash column chromatography were used without further purification. Bergenin was purchased from Sigma, vinyl esters were all from TCI America. Other acyl donors were synthesized at EnzyMed according to the following procedures.

Glutaric acid di(trifluoroethyl ester). Glutaryl chloride (10 ml, 76 mmol) was added dropwise to trifluoroethanol (27 ml, 370 mmol) in 150 ml of dry CH_2Cl_2, containing 40 ml of pyridine, at 0°C under argon. Reaction mixture was stirred for 30 min at 0° C and then overnight at room temperature. The mixture was extracted with 2 M HCl and the organic layer was washed by sodium bicarbonate and water and dried with sodium sulfate. Dichloromethane was evaporated and the product was purified by vacuum distillation (b.p. 70-71 °C/1 Torr) to give 19 g (86%) of pure ester. ^{1}H NMR ($CDCl_3$): δ 2.02 (m, 2H), 2.52 (t, J=7.2 Hz, 4H), 4.47 (q, J=8.6 Hz, 4H).

2-Pyrazinecarboxylic acid trifluoroethyl ester. The mixture of 2-pyrazinecarboxylic acid (1.2 g, 9.6 mmol), N,N'-dicyclohexylcarbodiimide (2.1 g, 10.2 mmol), and CF_3CH_2OH (5 ml, 168 mmol) in 50 ml of dry dichloromethane was stirred overnight at room temperature. The reaction mixture was then filtered and the precipitate washed with ethyl acetate. The ethyl acetate and dichloromethane solutions were combined and evaporated. The residue was purified by flash chromatography on silica gel using ethyl acetate/hexane mixture (3 : 1 v/v) as eluent, to give 1.5 g (78%) of pale yellow solid ester. ^{1}H NMR ($CDCl_3$): δ 4.85 (q, J=8.6 Hz, 2H), 8.81 (s, 1H), 8.84 (s, 1H), 9.35 (d, J=1.4 Hz, 1H).

Malonic acid di(trifluoroethyl ester). Ditrifluoroethyl malonate was synthesized in the same manner as 2-pyrazinecarboxylic acid trifluoroethyl ester with 75% yield. ^{1}H NMR ($CDCl_3$): δ 3.6 (s, 2H), 4.54 (q, J=8.6 Hz, 4H).

Butyl vinyl carbonate was synthesized using a published procedure.[26]

Acylation of Bergenin with Butyric Acid, Butyryl Chloride, and Benzoyl Chloride

The mixtures of bergenin with equimolar amounts of butyryl chloride or benzoyl chloride, or butyric acid and N,N'-dicyclohexylcarbodimide in dry pyridine were stirred overnight at room temperature. After solvent evaporation the dry residue was redissolved in methanol and analyzed by HPLC.

General Procedure for Enzymatic Reactions

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 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). Upon completion of the first acylation reaction, 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 the identical fashion as the first step. 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 and 50 mM of the appropriate acyl donor were used for the second step.

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 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 were recovered by evaporating the solvent in vacuum.

HPLC/MS Analysis

Reverse phase HPLC analyses were performed on a Shimadzu SIL-10 HPLC (photodiode array detection) using an μBondapak C18 column (Waters, 3.9x300 mm) and a water/acetonitrile linear gradient program (100% to 20% water over 30 min with a 1 ml/min flow rate). HPLC/MS analyses were done using a Perkin-Elmer LC200 and a PE-Sciex API100 electrospray MS (turbo-ion spray head) with the same gradient program, a Phenomenex IB-SIL C18 column (2x100 mm), and a flow rate of 0.4 ml/min. The positive mode mass spectrum was collected along with the UV absorbance at 275 nm.

NMR spectra

NMR spectra were recorded in DMSO-d_6 with tetramethylsilane as internal standard on a Bruker WM-360 spectrometer, ^{1}H spectra at 360 MHz and ^{13}C spectra at 90 MHz. Spectral assignments were made by comparing the spectra obtained with the published spectra of bergenin and its acetylated derivatives.[27,28] Acylated bergenin derivatives were purified for NMR spectroscopy by flash chromatography on silica gel using chloroform/methanol mixture (15 : 1 v/v) as eluent.

Bergenin-11-butyrate: ^{1}H NMR : δ (ppm) 0.91 (t, J=7.5 Hz, 3H, $\underline{CH_3}CH_2CH_2C{=}O$), 1.59 (q, J=7.2, 2H, $CH_3\underline{CH_2}CH_2C{=}O$), 2.34 (t, J=6.8 Hz, 2H, $CH_3CH_2\underline{CH_2}C{=}O$), 3.32 (t, J=9.0 Hz, 1H, C3-H), 3.69 (t, J=10.0 Hz, 1H, C4-H), 3.79 (s, 3H, C12-CH_3), 3.81 (m, 1H, C2-H), 4.01 (t, J=10.0 Hz, 1H, C4a-H), 4.13 (dd, J=11.8, 6.8 Hz, 1H, C11-H), 4.55 (d, J=11.8, 1H, C11-H), 5.02 (d, J=10.8 Hz, 1H, C10b-H), 5.65 (br.s., 1H, $OH_{alifat.}$), 5.73 (br.s., 1H, $OH_{alifat.}$), 7.03 (s, 1H, C7-H), 8.19 (s, 1H, $OH_{arom.}$), 9.80 (s, 1H, $OH_{arom.}$). ^{13}C NMR: δ 13.45 ($\underline{C}H_3CH_2CH_2C{=}O$), 18.00 ($CH_3\underline{C}H_2CH_2C{=}O$), 35.39 ($CH_3CH_2\underline{C}H_2C{=}O$), 59.78 (C12), 63.15 (C11), 70.26 (C3), 72.24 (C10b), 73.49 (C4), 78.64 (C4a), 81.60 (C12), 109.70 (C7), 115.80 (C10a), 118.2 (C6a), 140.60 (C9), 148.60 (C10), 151.0 (C8), 163.40 (C6), 172.80 ($CH_3CH_2CH_2\underline{C}{=}O$). MS: 399 ($M+H^+$).

Bergenin-4,11-dibutyrate: ^{1}H NMR : δ 0.90 (t, J=7.4Hz, 3H), 0.94 (t, J=7.3 Hz, 3H), 1.5 (m, 4H), 2.34 (t, J=7.2 Hz, 2H) 2.37 (t, J=7.2 Hz, 2H) - signals from two butyrates, 3.54 (m, 1H, C3-H), 3.77 (s, 3H, C12-CH_3), 3.92 (m, 1H, C2-H), 4.16 (dd, J=10.8, 6.2 Hz, 1H, C11-H), 4.29 (t, J=9.8 Hz, 1H, C4a-H), 4.51 (dd, J=10.4, 1.4, 1H, C11-H), 5.16 (d, J=10.5 Hz, 1H, C10b-H), 5.30 (t, J=9.3 Hz, 1H, C4-H), 5.81 (d, J=6.5, 1H, $OH_{alifat.}$), 6.99 (s, 1H, C7-H), 8.20 (s, 1H, $OH_{arom.}$), 9.8 (s, 1H, $OH_{arom.}$). ^{13}C NMR: δ 13.31 ($\underline{C}H_3CH_2CH_2C=O$), 13.37 ($\underline{C}H_3CH_2CH_2C=O$), 17.87 ($CH_3\underline{C}H_2CH_2C=O$), 18.03 ($CH_3\underline{C}H_2CH_2C=O$), 35.25 ($CH_3CH_2\underline{C}H_2C=O$), 35.49 ($CH_3CH_2\underline{C}H_2C=O$), 59.75 (C12), 62.67 (C11), 67.92 (C3), 71.90 (C10b), 73.84 (C4), 76.67 (C4a), 78.21 (C12), 109.70 (C7), 115.40 (C10a), 118.0 (C6a), 140.70 (C9), 148.0 (C10), 151.0 (C8), 162.70 (C6), 172.20 ($CH_3CH_2CH_2\underline{C}=O$), 172.70 ($CH_3CH_2CH_2\underline{C}=O$). MS:469 ($M+H^+$).

Bergenin-4-acetate-11-butyrate: ^{1}H NMR : δ 0.90 (t, J=7.4Hz, 3H, $\underline{CH_3}CH_2CH_2C=O$), 1.57 (q, J=7.3 Hz, 2H, $CH_3\underline{CH_2}CH_2C=O$), 2.12 (s, 3H, OAc), 2.34 (t, J=7.2 Hz, 2H, $CH_3CH_2\underline{CH_2}C=O$), 3.56 (m, 1H, C3-H), 3.78 (s, 3H, C12-CH_3), 3.91 (m, 1H, C2-H), 4.15 (dd, J=10.8, 6.2 Hz, 1H, C11-H), 4.29 (t, J=9.9 Hz, 1H, C4a-H), 4.50 (dd, J=10.4, 1.2, 1H, C11-H), 5.16 (d, J=10.5 Hz, 1H, C10b-H), 5.29 (t, J=9.3 Hz, 1H, C4-H), 5.87 (d, J=6.2, 1H, $OH_{alifat.}$), 6.99 (s, 1H, C7-H), 8.23 (s, 1H, $OH_{arom.}$), 9.84 (br. s, 1H, $OH_{arom.}$). ^{13}C NMR: δ 13.29 ($\underline{C}H_3CH_2CH_2C=O$), 17.80 ($CH_3\underline{C}H_2CH_2C=O$), 20.8 ($\underline{C}H_3C=O$), 35.15 ($CH_3CH_2\underline{C}H_2C=O$), 59.67 (C12), 62.57 (C11), 67.83 (C3), 71.81 (C10b), 74.03 (C4), 76.50 (C4a), 78.02 (C12), 109.60 (C7), 115.40 (C10a), 117.90 (C6a), 140.70 (C9), 147.90 (C10), 150.90 (C8), 162.70 (C6), 169.60 ($CH_3\underline{C}=O$), 172.70 ($CH_3CH_2CH_2\underline{C}=O$). MS: 441 ($M+H^+$).

ACKNOWLEDGMENTS

We thank Dr. John Krstenansky for helpful discussions, John M. Arnold for help with HPLC/MS measurements, and Dr. Nadezhda M. Astakhova for synthesizing acyl donors.

REFERENCES AND NOTES

1. Keinan, E.; Sinha, S. C.; Sinha-Bagch, A. *J. Chem. Soc. Perkins Trans. 1* **1991**, 3333-3339.
2. Patel, R. N.; Robison, R. S.; Szarka, L. J.; Kloss, J.; Thottathil, J.K.; Mueller, R.H. *Enzyme Microb. Technol* **1991**, *13*, 906-912.
3. Kieslich, K. Steroid Conversions. In *Economic Microbiology 5: Microbial Enzymes and Bioconversions*; Rose A. H., Ed.; Academic Press: London, 1980; pp. 369-465.
4. Kiener, A. US Patent 5,104,798, **1992**.
5. Delinck, D. L.; Margolin, A. L. *Tetrahedron Lett.* **1990**, *31*, 3093-3096.
6. Margolin, A. L.; Delinck, D. L.; Whalon, M. R. *J. Amer. Chem. Soc.* **1990**, *112*, 2849-2854.

7. Saksena, A. K.; Girijavallabhan, V. M.; Wang, H.; Liu, Y. T.; Pike, R. E.; Ganguly, A. K. *Tetrahedron Lett.* **1996**, *37*, 5657-5660.
8. Drauz, K.; Waldmann, H., Eds. *Enzyme Catalysis in Organic Synthesis: A Comprehensive Handbook;* VCH: Weinheim, 1995.
9. All products of bergenin acylation with lipases and subtilisin were purified to homogeneity and their structures were confirmed by ^{1}H- and ^{13}C-NMR and mass spectrometry (see Experimental section).
10. Verdine, G. L. *Nature*, **1996**, *384*, 11-13.
11. Michels, P. C.; Khmelnitsky, Yu. L.; Dordick, J. S.; Clark, D. S. *Trends Biotech.***1997**, in press.
12. Khmelnitsky, Yu. L.; Michels, P. C.; Dordick, J. S.; Clark, D. S. Generation of Solution-Phase Libraries of Organic Molecules by Combinatorial Biocatalysis. In *Molecular Diversity and Combinatorial Chemistry*; Chaiken, I. M.; Janda, K. D., Eds.; American Chemical Society: Washington, D. C., 1996; pp.144-157.
13. Alting-Mees, M. A.; Sorge, J. A.; Short, J. M. pBluescriptII: Multifunctional Cloning and Mapping Vectors. In *Recombinant DNA Methodology II*; Wu, R., Ed.; Academic Press: San Diego, 1995; pp. 171-183.
14. Macferrin, K. D.; Chen, L.;Terranova,M. P.; Schreiber, S. L.; Verdine, G. L. *Meth. Enzymol.* **1993**, *217*, 79-102.
15. Roessner, C. A.; Spencer, J. B.; Ozaki, S.; Min, C.; Atshaves, B. P.; Nayar, P.; Anousis, N.; Stolowich, N. J.; Holderman, M. T.; Scott, A. I. *Protein Expr. Purif.* **1995**, *6*, 155-163.
16. Fisher, C. W., Manjunath, S. S.; Estabrook, R. W. *Meth. Enzymol.* **1996**, *272*, 15-24.
17. Smith, M. C.; Furman, T. C.; Ignolia, T. D.; Pidgeon, C. *J. Biol. Chem.* **1988**, *263*, 7211-7215.
18. Stemmer, W. P. C. *Nature* **1994**, *370*, 389-391.
19. Kast, P.; Hilvert, D. *Pure Appl. Chem.*, **1996**, *68*, 2017-2024.
20. Moore, J. C.; Arnold, F. H. *Nature Biotechnol.* **1996**, *14*, 458-467.
21. Arnold, F. H. *Chem. Eng. Sci.* **1996**, *51*, 5091-5102.
22. Zhang, J. H.; Dawes, G.; Stemmer, W. P. C. *Proc. Nat. Acad. Sci. USA* **1997**, *94*, 4504-4509.
23. Jacobsen, J. R.; Hutchinson, C. R.; Cane, D. E.; Kholsa, C. *Science* **1997**, *277*, 367-369.
24. Svensson, I; Wehtje, E.; Adlercreutz, P.; Mattiasson, B. *Biotechnol. Bioeng.* **1994**, *44*, 549-556.
25. Khmelnitsky, Yu. L.; Welch, S. H.; Clark, D. S.; Dordick, J. S. *J. Amer. Chem. Soc.* **1994,** *116,* 2647-2648.
26. Pozo, M.; Pulido, R.; Gotor, V. *Tetrahedron* **1992,** *48,* 6477-6484.
27. Jahodar, L.; Kolb, I.; Lyska, A. *Fitoterapia* **1992,** 63, 260-261.
28. Ramaiah, P. A.; Row, L. R.; Reddy, D. S.; Anjaneyulu, A. S. R.; Ward, R. S.; Pelter, A. *J.Chem.Soc. Perkins Trans. 1* , **1979**, 2313-2316.