Chemo-Enzymatic Method for the Synthesis of Statine, Phenylstatine and Analogues

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Abstract: A chemo-enzymatic method for the synthesis of statine [(3*S*,4*R*)-4-amino-3-hydroxy-6-methylheptanoic acid] and statine analogues with natural and unnatural side chains is described. The key step of the synthesis is the diastereoselective reduction of a 2-alkyl-substituted 3-ketoglutarate by an NADPH-dependent ketoreductase. Two chiral centers are generated in one step, and high yields of a single stereoisomer are obtained (80–90% yield, 90–99% de). Subsequent chemical or enzymatic regio-selective hydrolysis to the mono-acid followed by rearrangement under Curtius or Hofmann conditions generates the final statines.

Keywords: biotechnology, dehydrogenase, enzyme, phenylstatine, statine

Natural and non-natural amino acids are present in a variety of natural and synthetic pharmaceutical compounds. In particular, substituted γ-alkyl β-hydroxy-γ-amino acids have been identified in a spectrum of natural products.[1–7] One of the first examples of a biologically-active compound containing β-hydroxy-γ-amino acids was the hexapeptide pepstatin, a potent carboxyl protease inhibitor, which contained two molecules of (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid also known as statine (Figure 1).[1] Recently, statine was reported to be an important part of a new class of triterpene derivatives with anti-HIV activity.[2,3] Since the discovery of statine, β-hydroxy-γ-amino acids have been identified in a variety of naturally occurring compounds with medicinal activity. Some examples include phenylstatine [(3*R*,4*S*)-5-phenyl-4-amino-3-hydroxypentanoic acid], which is part of the biologically-active compounds Hapalosin[4] and Dolastatin,[5] and isostatine, [(5*R*,4*R*,3*S*)-5-methyl-4-amino-3-hydroxyheptanoic acid], which is an essential amino acid in Didemnins,[6] a group of peptides with antitumor, antiviral and immunosuppressive activities (Figure 1).[7]

It is well known that minor changes in the structure or the absolute stereochemistry of a key component of a biologically-active compound can have an enormous impact in its *in vivo* activity. Such an effect was early observed in pepstatin, where the stereochemistry and alkyl substitution of statine, (present twice in a pepstatin molecule), played the most important role in its biological activity.[8] The anti-HIV compound RPR103611 was produced by the incorporation of statine into a natural product, and biological activity was increased. A similar effect was also observed when the (3*S*,4*S*)-statine side chain of RPR103611 was replaced with the (3*R*,4*S*)-diastereomer.[3]

In addition to a presence in various naturally-occurring compounds, statine and its analogues have potential application as components in new drug candidates. Only two of the diastereomeric statines are commercially available, and the cost reflects the difficulty of synthesis ($ 1450/gram). Considering the stereochemical diversity of the naturally-occurring statines and their analogues (Figure 1), and the demand for additional statine analogues that can be tested in pharmaceuticals, a simple, general synthetic strategy capable of generating each of the diastereomers of statines would be highly desirable.

We now report a straightforward, general chemo-enzymatic synthesis for both natural and non-natural statine analogues from readily available non-chiral starting materials. The method is illustrated by the synthesis of three β-hydroxy-γ-amino acids with natural side chains: statine, phenylstatine, and methylstatine (Table 1). The two key steps of the method involve a diastereoselective enzymatic reduction of a 2-alkyl-3-ketoglutarate di-ester, followed by the regio-selective hydrolysis to the mono-ester (Figure 2).

For identification of the best enzyme for the diastereoselective reduction of a starting 2-alkyl-3-ketoglutarate di-ester, the KRED Screening Set was utilized (KRED-10000, BioCatalytics, Inc., Pasadena, CA

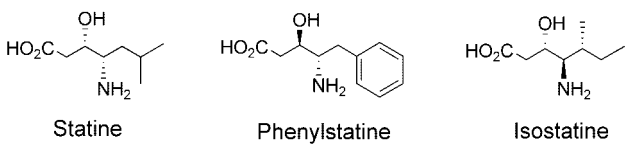

Figure 1. Structures of some β-hydroxy-γ-amino acids.

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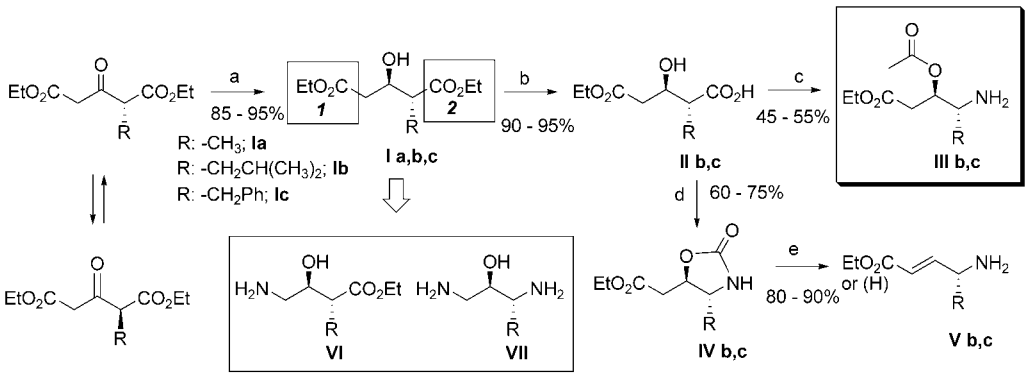


Figure 2. Synthetic scheme for the production of statines. *Reaction conditions:* a) 250 mM potassium phosphate (Kpi) pH 6.5, 100 mM NaCl, 5 mM NADPH, 2.5% (v/v) DMSO, 50 mM ketone substrate, 100 mM glucose, 2 mg/mL glucose dehydrogenase (GLDH), 5 mg/mL ketoreductase, 37 °C, 18 – 36 h; b) NaOH (4 equivs.) H_2O/EtOH (v/v 8/2), RT, 4 – 6 h then acidify (pH 2 – 3) with HCl, evaporate to dryness, add EtOH, filter, add HCl (cat) heat at 45 °C 12 – 18 h; c) i) CH_2Cl_2 Ac_2O (1.1 equivs.), TMSOTf (cat, 2% equiv.), 4 °C 15 – 30 min; ii) CH_2Cl_2, $(COCl)_2$ (1.2 equivs.), DMF (cat), RT 30 min, evaporate redissolve in THF, bubble NH_3 10 min, stir at 4 °C, 12 h or THF, *N*-methylmorpholine oxide (NMO, 1 equiv.), ethyl chloroformate (1 equiv.), – 18 °C, 20 min then bubble NH_3 for 10 min, stir overnight at 4 °C; iii) H_2O/CH_3CN (1/2, v/v) stir with $(CF_3CO_2)_2PhI$ (1.5 equivs.) at RT for 3 – 5 h; d) toluene, DPPA (1.1 equivs.), TEA (1.1 equivs.), 85 °C 2 – 3 h; e) NaOH (0.2 – 0.5 equiv.) H_2O/EtOH (v/v, 3/1) 40 – 50 °C, 2 – 4 h gives the free amino acid, or K_2CO_3 (0.5 equiv.), EtOH, 40 – 50 °C give the free amino ethyl ester. Other mild hydrolysis conditions gave the same product.

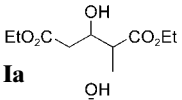
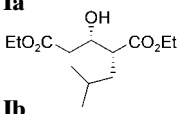
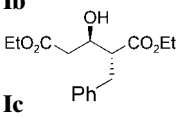
USA). The set contains 10 different ketoreductase enzymes for evaluation in the reduction of a ketone. In this case, 2-alkyl-3-ketoglutarate diethyl esters were prepared by the mono-alkylation of diethyl 3-ketoglutarate, and the product was screened against all 10 different ketoreductase enzymes.

The results are shown in Table 1. Due to the rapid isomerization of the two enantiomeric diethyl 2-alkyl-3-ketoglutarates, and because of the enzyme selectivity for reduction of only one, a single diastereomeric alcohol is obtained in high yields (**Ia**, **b**, **c** Figure 2 and Table 1). The two chiral centers that are present in the final statine as well as its absolute stereochemistry are both determined in this first enzymatic step.

The second key step of the synthesis involves a chemical regioselective hydrolysis of each enzymatically-produced diethyl 2-alkyl-3-hydroxyglutarate (**I**, Figure 2). Early experiments revealed a significant difference in the chemical hydrolysis rates between the two esters ***1*** and ***2*** (**I**, Figure 2) regardless of the alkyl substitution at the 2-position (phenyl or isobutyl). More specifically, incubation for 30 min of diethyl 2-alkyl-3-hydroxyglutarate in an aqueous/ethanol (8/2, v/v) solution containing a 50% excess of sodium hydroxide, gave complete hydrolysis of the ester group ***1*** producing the mono-acid as the only product. More severe conditions (4 equivs. NaOH, 4 – 6 h) were required for the formation of the di-acids. Based on this result and the microscopic reversibility of organic reactions we speculated that esterification of the di-acid would first proceed to carboxylic acid ***1*** giving the mono-ester **II** (Figure 2). Indeed, in every case studied, when the di-acid was stirred in ethanol in the presence of catalytic amounts of hydrochloric acid at 45 °C, formation of the mono-ethyl 2-alkyl-3-hydroxyglutarate **II** (Figure 2) was produced in quantitative yields during the first 10 – 14 h. Chiral analysis of the mono-acid (**II**, Figure 2) showed no isomerization of either asymmetric carbon. The optical purity is thus retained after this hydrolysis-esterification sequence.

In all the substrates that we have examined to date, the previously described chemical hydrolysis/esterification sequence has proven to be a general method for the synthesis of mono-esters **II** (Figure 2). However, other approaches relying on the use of enzymes for hydrolysis are also applicable. In this regard we tested the enzymatic hydrolysis of substrates **I** (Figure 2) using a set of twenty-four commercially-available lipases (ICR Screening kit available from BioCatalytics Inc., Pasadena, CA). In every case studied, a minimum of three enzymes were able to catalyze the hydrolysis of the ester ***1*** (**I**, Figure 2) of each diethyl 2-alkyl-3-hydroxyglutarate giving the same product as the one obtained under the mild chemical hydrolysis conditions. Therefore, an enzymatic esterification of the chemically produced di-acid to form the less hindered mono-ester **II** (Figure 2) is an alternative method for chemo-selective esterification. Introducing a second enzymatic step in the synthesis can potentially increase the optical purity of the final statine in cases where small diastereomeric impurities are present from the first reduction step (Table 1). For example, lipase ICR112 selectively hydrolyzed the contaminating diastereomers of diethyl 2-methyl-3-hydroxyglutarate (**Ia**, Figure 2), produced by KRED-108 reduction (Table 1), and increased the diastereomeric purity of the starting material from

Table 1. Reaction yields and diastereomeric purities of the enzymatic reduction of diethyl 2-alkyl-3-ketoglutarates giving **Ia, b, c** as shown in Figure 2.

Product[c]	Yield[a] (A/B/C/D)[b]		
	KRED-101	KRED-107	KRED-108
 **Ia**	> 97% (65/0/9/26)	55% (4.5/4.5/91/0)	90% (5.5/5.5/89/0)
 **Ib**	95% (0/5/0/95)		
 **Ic**	10% (28/27/45/0)		> 97% (0/ > 99/0/0)

[a] Yield was based in small-scale reactions (2 mL) where substrate (40 mM) was mixed with NADPH (10 mM), GLDH (2 mg/mL), KRED (5 mg/mL), glucose (100 mM), DMSO (2.5%, v/v), and potassium phosphate (Kpi, 250 mM, pH 6.5) and was incubated for 14 h at 37 °C. It was calculated by GC analysis of crude reaction extracts.

[b] Ratio of diastereomers, A, B, C, D according to their retention time on a chiral GC column (details in Experimental).

[c] The major diastereomer is shown. For **Ib** (coming from KRED-101 reduction) is (3*S*,4*R*), and for **Ic** (coming from KRED-108 reduction) is (3*R*,4*R*). The absolute stereochemistry of the major diastereomers for **Ia** was not identified.

78% to more than 99%. The recovery of the pure diastereomer from the mixture was in the range of 90 to 95%.

Chemical rearrangement of the mono-acid **II** (Figure 2) to the amine was the last step of the synthesis. One of the simplest and most straightforward methods for the formation of an amine from a carboxylic acid consists of a Curtius rearrangement that is performed in one step using the reagent diphenylphosphoryl azide (DPPA).[9] Heating the acid with DPPA in toluene and in the presence of triethylamine forms an intermediate isocyanate, which upon addition of an alcohol, is trapped to the corresponding carbamate. When *tert*-butyl alcohol is utilized as the alcohol the Boc-protected amine is thus obtained.[9] When these conditions were applied for the rearrangement of each mono-acid **II**, the cyclic carbamates **IV** instead of the Boc-protected amines were obtained in high yields (Figure 2). The same products were predominantly forming even when *tert*-butyl alcohol instead of toluene was used as the solvent for the rearrangement, or when an aqueous/organic mixture was used for rearranging the corresponding amides under Hoffman conditions using [bis(trifluoroacetoxy)iodo]benzene [$(CF_3CO_2)_2PhI$].[10] These results indicate that regardless of its formation, the intermediate isocyanate reacts preferentially with the adjacent free β-hydroxy group in an intramolecular reaction to form the cyclic carbamate **IV** (Figure 2). Contrary to a published report,[11] hydrolysis of the cyclic carbamates **IV** of either statine or phenylstatine produced the dehydrated amines **V** (Figure 2) instead of the expected free amino alcohols. Various conditions[12] for the hydrolysis were investigated including incubation with catalytic amounts of various bases such as NaOH, K_2CO_3, LiOH, $CsCO_3$ or reaction with $LiOH/H_2O_2$. In all cases either no reaction occurred or the dehydrated compound **V** (Figure 2) was the predominant product. Although not the intended product, the α,β-unsaturated-γ-amino acid **V** that is produced by the hydrolysis of the cyclic carbamates of statine and phenylstatine represents by itself a potentially interesting chiral synthon.

In the absence of a simple hydrolysis methodology to the synthesis of statines from their cyclic carbamate precursors, an alternative route was taken. Protection of the alcohol prior to rearrangement was a successful strategy for obtaining the free amino alcohol. Among various protecting groups and reaction conditions that were investigated, the simplest and highest yielding approach relied on the formation of an acetate by reacting the alcohol with one equivalent of acetic anhydride in methylene chloride and in the presence of catalytic amounts (1–2% relative to alcohol) of trimethylsilyl trifluoromethanesulfonate (TMSOTf).[13] The temperature must be maintained at 4 °C to eliminate formation of other by-products that decrease the reaction yield. Under these conditions the alcohol was protected as the acetyl ester in yields greater than 95%.

Rearrangement to the final amine was accomplished using two different methods. In one method the protected amine was rearranged with DPPA in toluene, however, low yields were obtained. Although many other conditions remain to be investigated, rearrangement to the free amine was achieved easily after formation of the amide and then reaction at room temperature with 1.5 equivalents of [bis(trifluoroacetoxy)iodo]benzene for three hours in an aqueous/acetonitrile mixture.[10] Product isolation was accomplished by evaporation of the acetonitrile from the reaction mixture followed by the addition of HCl (3 M), diethyl ether and extraction. The amine forms the hydrochloride salt and moves into the aqueous layer and all other organic by-products are extracted into the organic layer. Evaporation of the aqueous solution to dryness gave a white powder of very clean statine analogues in yields ranging from 45 to 55% calculated from the mono-acid **II** (Figure 2). The absolute stereochemistry and enantiomeric purity of the mono-acid **II** is retained in to the final statine **III** (Figure 2) as shown by chiral analysis of the final amino acids.

The absolute stereochemistry of each statine was identified using both chiral chromatography and

^{1}H NMR analysis. It is known, for example, that the coupling constants between protons at carbons C-3 and C-4 of the cyclic carbamates **IV** (Figure 2) are 5 Hz when they possess the *trans* configuration and 8.0 Hz when they possess the *cis* configuration.[8] Phenylstatine cyclic carbamate **IV** showed coupling constants between 4.7 – 5 Hz indicating absolute stereochemistry of either (3*S*,4*S*) or (3*R*,4*R*). Statine chemical shift correlations were less definitive, so its stereochemistry was assigned by chiral chromatography and comparison with authentic material. Optical rotation analysis of the final statines showed a positive rotation for both the statine and phenylstatine. These results indicated (3*R*,4*R*) stereochemistry for the phenylstatine and (3*R*,4*R*) or (3*S*,4*R*) stereochemistry for statine. The optical purity and absolute stereochemistry of both statines was finally confirmed using chiral GC or HPLC analysis by comparison with standard mixtures of known statine diastereomeric mixtures that were synthesized using reported methodology.[14a] The absolute stereochemistry of statine was finally assigned as the (3*S*,4*R*). Comparisons between the optical purities of the final statines and the enzymatic reductions on the first step of our synthesis indicated that no isomerization occurred during the hydrolysis or the rearrangement.

Various chemical syntheses of β-hydroxy-γ-amino acid analogues, mainly statine, phenylstatine and isostatine, have been published in the recent literature. Almost all reported methodologies however, begin with a chiral starting material, usually a natural α-amino acid analogue.[14] More recently, synthetic approaches that utilize other chiral, or non-chiral synthons have been reported.[15] Besides the low diastereomeric purities of product that were sometimes obtained by these methods, long reaction sequences, chiral catalysts, use of expensive reagents and stringent reaction conditions are usually employed. Even more importantly, none of these methods is generally-applicable for the synthesis of all four diastereomers of each statine analogue, and very few methodologies can generate statines with non-natural amino acid side chains.[15a, c]

The synthesis of statine analogues that is described in this report has many advantages over the previously published chemical methodologies. It is short, high-yielding and consists of simple reactions mostly in aqueous media that can be easily scaled to larger quantities. The starting materials for the synthesis of the diethyl 2-alkyl-3-ketoglutarates are inexpensive, non-chiral, and readily available consisting of the diethyl 1,3-acetonedicarboxylate and the appropriate alkyl halide. We have synthesized various alkyl-substituted ketoglutarates by treatment of diethyl 1,3-acetonedicarboxylate with LDA followed by addition of the alkyl halide in yields varying from 50 to 85% depending on substrate and reaction conditions. Statine analogues with various stereochemistries carrying both natural and unnatural alkyl substitutions are accessible using this method. Furthermore, other potentially useful compounds including 2-alkyl-3-hydroxy-4-aminobutanes (**VI**, Figure 2) and 1-alkyl-2-hydroxy-1,3-diaminopropanes (**VII**, Figure 2) can be synthesized using the same chemical methodology from the respective rearrangement of the less substituted mono-ethyl 2-alkyl-3-hydroxyglutarate or from the corresponding di-acids. The diamine analogue of phenylstatine (**VII**, R = benzyl, Figure 2), for example, is the central component of the commercial antiviral and HIV drug Amprenavir.[16] Formation of these analogues as well as synthesis of other diethyl 2-alkyl-3-ketoglutarates bearing unnatural alkyl substitutions is under investigation.

Probably the best feature of this synthesis is that the key step, which is the reduction of 2-alkyl-3-ketoglutarates, is performed by an enzyme. Using enzymes as catalysts for organic reactions has the advantage of potential modification or improvement of their substrate specificity and stereoselectivity by directed evolution techniques.[17] These methodologies are lately becoming a more routine practice for the modification of a spectrum of enzymatic properties (substrate range, strereoselectivity, thermal stability are only a few examples). In addition, when their corresponding DNA has been cloned, large amounts of catalyst can be easily produced by fermentation of recombinant bacteria that express the gene. Work on the discovery of new enzymes that would accept a wider variety of substrates and give various diastereomers is already under way.

Experimental Section

Synthesis of Diethyl 2-Alkyl-3-ketoglutarates

A typical protocol for the synthesis of both diethyl 2-phenyl- and 2-isopropyl-3-ketoglutarates is described. Under a nitrogen atmosphere diethyl 1,3-acetone-dicarboxylate (15 mL, 0.072 mol) was dissolved in tetrahydrofuran (60 mL) and the mixture was cooled at $-18\,^{\circ}C$ (ice/NaCl) for 10 minutes before lithium diisopropylamine (74 mL, 2 M in hexane, 0.148 mol) was slowly added over a period of 20 minutes. After stirring the solution at $-18\,^{\circ}C$ for 10 minutes, a THF solution (20 mL) containing benzyl bromide (9.5 mL, 0.080 mol) or 1-iodo-2-methylpropane (0.08 mol) was slowly added, and the solution was stirred for another 2.5 h (temperature was slowly increased to $-10\,^{\circ}C$) and 2 more hours at room temperature before complete reaction was observed by thin layer chromatography analysis of reaction aliquots. The reaction mixture was then poured into an ice-cold aqueous hydrochloric acid solution (100 mL of 2 M HCl) and was extracted twice with ethyl acetate (200 mL × 2). The combined organic layers were back-extracted with brine (50 mL), dried over Na_2SO_4, and evaporated to dryness giving 25 g of an oily product. This product can be enzymatically reduced to the alcohol without any further purification. Pure diethyl 2-benzyl- or 2-isobutyl-3-ketoglutarates were obtained using silica gel chromatography (hexane/EtOAc, v/v, 8/2, 70% to 85% isolated yield).

Enzymatic Reductions (Ia, b, c, Figure 2)

The Ketoreductase Screening Set (KRED-10000; BioCatalytics, Inc., Pasadena, CA USA) containing ten different ketoreductases was screened to determine the best enzyme for the diastereoselective reduction of every diethyl 2-alkyl-3-ketoglutarate. In addition to the 10 ketoreductases both NADPH and glucose dehydrogenase (GLDH) are products available from BioCatalytics. Individual reactions containing each ketoreductase (2 mg/mL), NADPH (5 mM), NaCl (100 mM) DMSO (2.5% or 5%, v/v) each substrate (25 mM), glucose (100 mM) and glucose dehydrogenase (GLDH, 2 mg/mL) for cofactor recycling were prepared in a phosphate-buffered (1 mL, 300 mM, pH 6.5) solution. The reactions were incubated at 37 °C overnight before they were extracted with ethyl acetate and analyzed by GC or HPLC chromatography.

Larger-scale enzymatic reductions were prepared according to the following protocol. A phosphate-buffered solution (250 mL, pH 6.7, 200 mM) containing NaCl (200 mM), glucose (100 mM), NADPH (0.1 g, 0.5 mM), lyophilized glucose dehydrogenase (100 mg) and the appropriate ketoreductase (150 mg) was mixed with a DMSO solution (10 mL) containing diethyl 2-benzyl- or 2-isobutyl-3-ketoglutarate substrates (1.3 M) giving a final substrate concentration of 50 mM. The reaction mixture was stirred at 37 °C until HPLC or GC analysis of crude extracts showed complete reaction, usually occurring after two days. Periodically the pH was readjusted to 6.7 with NaOH (2.5 M) addition and a second batch of NADPH (0.1 g) was added after the first day of reaction. Product was isolated by extracting the crude reaction mixture with ethyl acetate (150 mL × 2). Some times centrifugation (6,000 rpm, 10 min) was required for the aqueous and the ethyl acetate layers to separate as clear solutions. The combined organic layers were back-extracted with brine, dried over Na_2SO_4 and evaporated to dryness. Pure alcohols (85% to 95% yield) were isolated after silica gel chromatography (80/20, v/v, hexane/EtOAc). The optical purity of each product was determined by chiral GC chromatography using a ChiralDex column (Chiral Technologies, 130 °C 2 min and then to 180 °C, 0.5 or 1 °C/min) after each enzymatically-produced alcohol was derivatized as the triflate ester. Products were analyzed by NMR spectroscopy.

Ia (from KRED-108): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.2 (dd, 3H, -CH_3), 2.3 (t, 6H, $CO_2CH_2CH_3$), 2.58 (m, 3H, $CH(H_2)$-$CO_2C_2H_5$), 4.18 (5H, $CO_2CH_2CH_3$ and C*H*-OH).

Ib (from KRED-101): ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.92 (t, 6H, $CH_2CH(CH_3)_2$), 1.26 (two overlapping triplets, 6H, $CO_2CH_2CH_3$ esters ***1*** + ***2***, Figure 2), 1.65 (broad multiplet, 3H, $CH_2CH(CH_3)_2$), 2.52 (dd, dd second dd overlaps with multiplet, 3H, $CH_2CO_2C_2H_5$ ABX, and $CHCO_2C_2H_5$), 4.18 (m, 5H, C*H*-OH, $CO_2CH_2CH_3$ esters ***1*** + ***2***, Figure 2).

Ic (From KRED-108): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.11 (t, 3H, $CO_2CH_2CH_3$, ester ***2***, Figure 2); 1.27 (t, 3H, $CO_2CH_2CH_3$, ester ***1***, Figure 2), 2.57 (d, s 2H, ABX, CH_2-$CO_2C_2H_5$), 2.80 (m, 1H, C*H*-$CO_2C_2H_5$), 3.0 (m, CH_2-Ph), 4.09 (q, 2H, $CO_2CH_2CH_3$ ester ***2***, Figure 2), 4.18 (q, m 3H, $CO_2CH_2CH_3$ ester ***1***, Figure 2, and C*H*-OH), 7.25 (m, 5H, CH_2-*Ph*).

Chemical and Enzymatic Hydrolysis to Each Mono-Ester (II, Figure 2).

Each diethyl 2-benzyl- or 2-isobutyl-3-hydroxyglutarate (**IIb, c** Figure 2) (50 mM) was stirred in an aqueous/ethanol (v/v, 8/2) solution containing NaOH (75 mM) for 30 to 45 min at room temperature. The reaction mixture was then acidified to pH 2.5 – 3 with HCl addition (2 M), evaporated to half the original volume and extracted with ethyl acetate (× 2). Back-extraction of the combined organic layers with brine, drying over Na_2SO_4 and evaporation to dryness gave quantitative yields of the mono-ethyl- 2-benzyl or 2-isobutyl-3-hydroxyglutarate where ester ***1***, of either **Ib**, **c** (Figure 2) was selectively hydrolyzed.

Regio-selective hydrolysis of the ester ***2*** (Figure 2) to give **IIb, c** was accomplished according to the following sequence. Each di-ester **I** (R = benzyl, isobutyl, **Ib**, **c** Figure 2) (0.6 M) was stirred at RT with NaOH (2.5 M) for 4 – 6 h in an aqueous/ethanol (20 mL, 8/2, v/v) solution until complete hydrolysis to the di-acid was observed. The solution was acidified to pH 2 to 2.5 with HCl (2.5 M) and evaporated to dryness. The remaining solid was vigorously stirred with 40 mL ethanol and the insoluble salts (NaCl) were removed by filtration. After washing the salts with another 50 mL of ethanol, the organic solutions were combined and evaporated to half volume. Two drops of concentrated HCl were then added to the medium and the mixture was stirred at 45 – 50 °C for 18 to 24 h until complete conversion to the mono-acid was observed. Evaporation of the ethanol solvent gave very clean mono-ester **IIb**, **c** (hydrolysis of ester ***2***, **Ib**, **c** Figure 2) (> 95% yield), which was used in the next step without any further purification. Samples were analyzed by ^{1}H NMR spectroscopy.

IIc: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.23 (t, 3H $CO_2CH_2CH_3$, ester ***1***, Figure 2), 2.6 (m, 2H CH_2-$CO_2C_2H_5$), 2.8 (m, 1H, C*H*-$CO_2C_2H_5$), 3.05 (m, CH_2-Ph), 4.18 (q, m 3H, $CO_2CH_2CH_3$ and C*H*-OH), 7.25 (m, 5H, CH_2-*Ph*).

For **IIb** the correct hydrolysis product was assigned after rearrangement with DPPA to form the cyclic carbamate **IVb** (Figure 2) and ^{1}H NMR analysis of this compound (*vide infra*).

Enzymatic reactions were performed in aqueous potassium phosphate-buffered solutions (pH 7, 250 mM) containing 2 mg/mL of each lipase. An ICR Lipase Screening Set (BioCatalytics, Inc., Pasadena, CA) consisting of twenty-five enzymes was used to screen for a hydrolytic enzyme capable of catalyzing the regioselective hydrolysis of each enzymatically-produced diethyl 2-alkyl-3-hydroxyketoglutarate. Each of the 25 hydrolytic enzymes in the Chirazyme Screening Set (1 mg/mL) was incubated at 37 °C in a potassium phosphate buffer solution (250 mM, pH 7) containing substrate (40 mM) and DMSO (5%, v/v). At certain time increments a sample was taken, acidified with HCl, extracted with diethyl ether, mixed with CH_2N_2 and analyzed by chiral GC chromatography. The enzymatic products are identical to those obtained under the mild hydrolysis conditions (hydrolysis of ester ***1*** of **Ib** and **Ic** Figure 2).

Protection of the Alcohol and Rearrangement with [Bis(trifluoroacetoxy)iodo]benzene [$(CF_3CO_2)_2PhI$], (IIIb, c Figure 2).

In CH_2Cl_2 (10 mL) ethyl 2-isobutyl-3-hydroxy glutarate (**IIb**, Figure 2) (0.8 g, 3.5 mmol) was dissolved and the solution was stirred in an ice bath for 15 min before the addition of acetic anhydride (0.4 mL, 4 mmol) and trimethylsilyl trifluoromethanesulfonate (TMSOTf) (15 μL, 0.07 mmol). The reaction mixture was stirred for 1–2 h at 4 °C, mixed with EtOAc (20 mL) washed with HCl (0.1 M, 5 mL) and evaporated to dryness giving the acetylated alcohol as the only detectable product (0.8 g 90% yield).

The protected alcohol (0.3 g, 1.3 mmol) redissolved in CH_2Cl_2 (5 mL) containing one drop of DMF and oxalyl chloride (0.2 mL, 2.3 mmol). After stirring the reaction at room temperature for 30 min the solvent was evaporated to dryness, the oily mixture was redissolved in THF (10 mL), and cooled in an ice-bath for 10 min. Ammonia gas was bubbled for 30 min and the reaction mixture was stirred for another 12 h at 4 °C in a tightly closed vessel. Rotary evaporation to dryness gave an oily residue that was dissolved into an aqueous/acetonitrile (v/v, 1/1, 4 mL) solution containing [$(CF_3CO_2)_2Ph$] (0.77 g, 1.8 mmol). The mixtures was stirred at RT for 3.5 h, concentrated to half volume by rotary evaporation, mixed with HCl (4 mL, 6M) and extracted with diethyl ether (5 mL). Evaporation to dryness of the aqueous layer gave pure statine (**III**, R = isobutyl, Figure 2) (0.14 g, 47% yield).

IIIb: ^{1}H NMR (400 MHz, D_2O): δ = 0.94 (dd, 6H, $CH_2CH(CH_3)_2$) 1.25 (t, 3H, $CO_2CH_2CH_3$), 1.55 (m, 2H, $CH_2CH(CH_3)_2$), 1.75 (hept, 1H, $CH_2CH(CH_3)_2$), 2.15 (s, 3H, CH-OCOC*H*$_3$), 2.86 (dd, 2H, ABX, J = 3.99, 1.99 Hz, $CH_2CO_2C_2H_5$), 3.62 (hex, 1H, C*H*-NH_2), 4.18 (q, 2H, $CO_2CH_2CH_3$), 5.4 (m, 1H, C*H*-$OCOCH_3$). HPLC-MS analysis (ESI positive ion) showed a single peak (column: C8, elution solvent H_2O/MeOH, v/v, 60/40 isocratic) with *m/z* (M + 1) = 246.0 ($C_{12}H_{23}NO_4$, calculated mass 245.1). HPLC-MS instrument: ThermoFinnigan LCQ advantage.

Use of the same reaction sequence for rearranging the acetyl-protected alcohol of the diethyl 2-benzyl-3-hydroxyglutarate (**IIc**, Figure 2) gave an overall yield of 60% to the phenylstatine **IIIc** (Figure 2).

IIIc: ^{1}H NMR (400 MHz, D_2O): δ = 1.21 (t, 3H, $CO_2CH_2CH_3$), 2.17 (s, 3H, CH-OCOC*H*$_3$), 2.86 (dd, 2H, J = 5.59, 3.99 Hz, $CH_2CO_2C_2H_5$), 3.01 + 3.08 (dd + dd, CH_2Ph), 3.92 (hex, 1H, C*H*-NH_2), 4.13 (q, 2H, $CO_2CH_2CH_3$), 5.31 (m, 1H, C*H*-$OCOCH_3$), 7.4 (m, 5H, CH_2*Ph*). HPLC-MS analysis (ESI positive ion) showed a single peak (column: C8, elution solvent H_2O/MeOH, v/v, 60/40 isocratic) with *m/z* (M + 1) = 280.2 ($C_{15}H_{21}NO_4$, calculated mass 279.15). HPLC-MS instrument: ThermoFinnigan LCQ advantage.

The absolute stereochemistry and optical purity of both statines (both had a positive optical rotation) was assigned using chiral GC and HPLC analysis by comparison with authentic material that was synthesized under known chemical methodology.[14a] Under these conditions starting from the *N*-Boc protected *R*- or *S*-leucine or phenylalanine the 4*R*- or 4*S*-*N*-Boc-protected 3-keto ester precursors of statine and phenylstatine were chemically prepared and were then reduced to the *N*-Boc protected statines using sodium borohydride, which gives products of a known diastereomeric nature.[14a] For example, the ethyl 4*S*-*N*-Boc-3-ketostatine gives after reduction a 70/30 mixture of the (3*R*,4*S*)- and (3*S*,4*S*)-statines. The other set of statine diastereomers was synthesized from the 4*R*-*N*-Boc-3-ketostatine. Chiral GC analysis (Varian, ChiralSil Val-L column, 160 °C or 180 °C isocratic) separated all four diastereomers. Compounds **IIIb**, **c** reacted in EtOH with di-*tert*-butyl dicarbonate (Boc_2O) in the presence of triethylamine. Under these conditions the amine was protected with the Boc group and the alcohol was deprotected giving essentially the same compounds as the chemically synthesized standards. The GC retention times between the standards and our statines were then compared.

Rearrangement with Diphenylphosphoryl Azide (DPPA) to IVb, c and Vb, c (Figure 2)

In toluene (30 mL) each mono-acid **IIb**, **c** (7.6 mmol) (Figure 2) was dissolved. The mixture was heated at 85 °C before DPPA (2 mL, 9.3 mmol) and triethylamine (0.8 mL, 7.8 mmol) were added in one portion. The solution was stirred at this temperature for 2 h and another 3 h at 65 °C. At the end of the reaction, ethyl acetate (30 mL) was added into the reaction mixture and the organic layer extracted once with HCl (20 mL, 0.5 M), saturated $NaHCO_3$ (20 mL) and brine (20 mL). Drying over Na_2SO_4 and evaporation of the organic solvents gave an oily precipitate that consisted mainly of the cyclic carbamates **IV** (Figure 2). Pure carbamates were obtained after silica gel purification (60% to 70% isolated yield).

IVb: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.92 (t, 6H $CH_2CH(CH_3)_2$), 1.27 (t, 3H, $CO_2CH_2CH_3$), 1.45 + 1.51 (m + m 2H, $CH_2CH(CH_3)_2$), 1.65 (hept, 1H $CH_2CH(CH_3)_2$), 2.65 + 2.78 (dd + dd, ABX, CH_2-$CO_2C_2H_5$), 3.59 (pent, 1H C*H*-NHCO), 4.18 (q, 2H, $CO_2CH_2CH_3$), 4.57 (q, 1H, C*H*-OCO).

IVc: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.27 (t, 3H, $CO_2CH_2CH_3$), 2.6 + 2.75 (dd + dd, ABX, 2H, CH_2-$CO_2C_2H_5$), 2.82 + 3.0 (dd + dd, ABX, 2H CH_2-Ph), 3.81 (pent, 1H, C*H*-NHCO), 4.18 (q, 2H, $CO_2CH_2CH_3$), 4.7 (q, 1H, C*H*-OCO), 5.25 (s, 1H N*H*), 7.3 (m, 5H, CH_2-*Ph*); ^{13}C NMR (100 MHz, $CDCl_3$): δ=11.136 (s, $CO_2CH_2CH_3$), 39.123 (s, CH_2-$CO_2C_2H_5$), 41.6 (s, CH_2-Ph), 58.819 (s, $CO_2CH_2CH_3$), 61.175 (s, *C*H-OCO), 76.706 + 77.032 + 77.237 + 77.347 (*C*DCl_3), 77.540 (s, *C*H-NH), 127.382 + 129.083 + 135.892 (CH-*Ph*), 157.55 (s, O-*C*O-N), 169.231 (s, *C*$O_2C_2H_5$); MS analysis: $C_{14}H_{17}O_4N$ (MALDI-FTMS), M + Na^+ = 286.105 (expected: 286.105).

The crude oily product can be hydrolyzed to the α,β unsaturated amine (**Vb**, **c**, Figure 2) without prior purification. In an aqueous/ethanol solution (10 mL, 9/1, v/v) the crude cyclic carbamate product (0.7 M) was mixed with NaOH (2.5 M) and the solution was stirred at 50 °C for 3 h. Extraction of the basic aqueous solution with diethyl ether, acidifying with HCl (2 M, pH 4-5) and evaporation to dryness gave a white precipitate. Addition of ethanol to this precipitate followed by vigorous stirring, filtration to remove the insoluble NaCl salts and solvent evaporation to dryness gave a white powder consisting of very clean unsaturated amino acids **Vb**, **c** (Figure 2) (80% to 90% isolated yield).

Vb: ^{1}H NMR (400 MHz, D_2O, CD_3CO_2D): δ = 0.96 (t, 6H, $CH_2CH(CH_3)_2$), 1.62 (m, 3H, $CH_2CH(CH_3)_2$), 4.08 (q, 1H, C*H*-NH_2), 6.17 (d, 1H, J = 15.59 Hz, CH=C*H*-$CO_2C_2H_5$), 6.77 (dd, 1H, J = 8.39, 7.99, C*H*=CH-$CO_2C_2H_5$); ^{13}C NMR (100 MHz, D_2O, CD_3CO_2D): δ = 21.858 + 23.05 (s + s, $CH_2CH(CH_3)_2$),

25.037 (s, $CH_2CH(CH_3)_2$), 41.971 (s, $CH_2CH(CH_3)_2$), 51.717 (s, *C*H-NH_2), 127.277 (s, CH=*C*H-$CO_2C_2H_5$), 142.906 (s, *C*H=CH-$CO_2C_2H_5$), 170.686 (s, CO_2H).

Vc: ^{1}H NMR (400 MHz, D_2O): $\delta = 2.78 + 2.9$ (dd + dd, AB, 2H, CH_2Ph), 3.8 (q, 1H, C*H*-NH_2), 5.8 (d, 1H, $J = 16$ Hz, CH=C*H*-$CO_2C_2H_5$), 6.6 (dd, 1H, $J = 6.39$, 6.39 Hz, C*H*=CH-$CO_2C_2H_5$), 7.38 (m, 5H, CH_2*Ph*); ^{13}C NMR (100 MHz, D_2O): δ=42.276 (s, *C*HPh), 53.33 (s, *C*H-NH_2), 126.292 + 126.895 + 128.836 + 129.897 + 138.425 + 145.52 (s, CH*Ph* + *C*H=*C*H-$CO_2C_2H_5$), 175.551 (s, CO_2H).

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