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Chemoenzymatic synthesis of ketomethylene tripeptide isosteres

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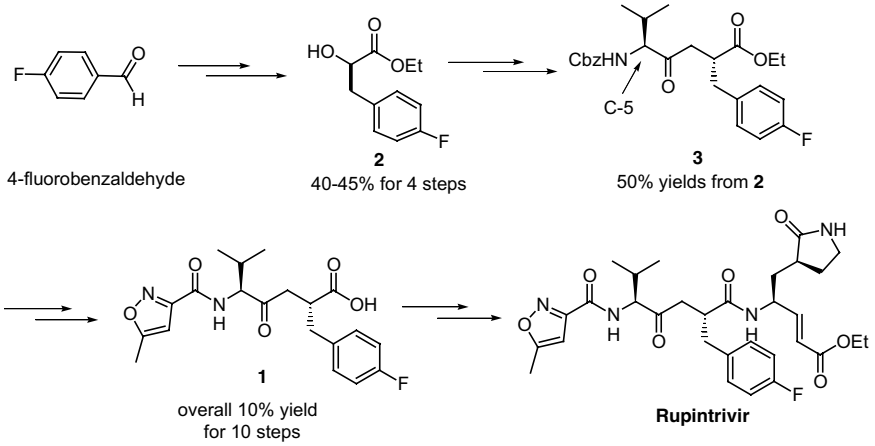
Abstract—A chemoenzymatic method is described for the synthesis of a desired ketomethylene tripeptide isostere. The key step is an enzymatic hydrolysis, which removes the C-terminal ester-protecting group under mild conditions without epimerizing the existing stereogenic center and produces the desired stereoisomer in high enantiomeric excess (98% de, 97% ee). The method is short with overall 15–20% yields after seven synthetic steps from readily available starting materials being obtained.

1. Introduction

Ketomethylene peptide isosteres, where a scissile amide bond is replaced with a nonscissile carbon–carbon bond, are recurring motifs used to design a variety of therapeutic compounds with enhanced stability toward proteolysis.[1–9] For example, Rupintrivir™, which contains ketomethylene tripeptide isostere **1** (Scheme 1), is a lead candidate entering human clinical trials to treat the common cold infection.[7]

A number of methods have been reported for the preparation of ketomethylene peptide isosteres with the desired stereochemical configuration.[1–10] Among them, the shortest approach uses a chiral alkylation strategy to produce both C- and N-terminal masked ketomethylene dipeptide isosteres from amino acids and (*R*)-2-hydroxy carboxylic esters.[10] Assuming mild deprotection conditions can be identified, these building blocks can then be potentially incorporated into longer peptide sequences such as tripeptide isosteres (e.g., **1**) and peptide mimetics. For the synthesis of **1** and ultimately Rupintrivir™ (Scheme 1),[11] this methodology is faced with several issues. First of all, the desired enantiomerically pure (*R*)-3-(4-fluorophenyl)-2-hydroxypropionic ester **2** is not commercially available and its synthesis requires



Scheme 1.

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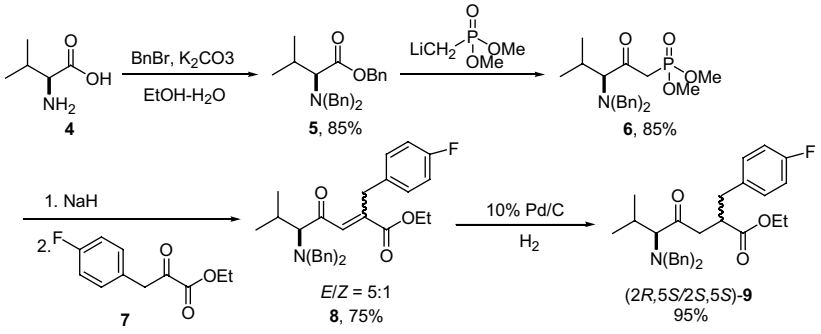
four steps with overall only 40–45% yields from 4-fluorobenzaldehyde.[12] Secondly, the chiral alkylation step usually gives poor yields (40–52%).[10] After extensive optimization, the best yield obtained for the dipeptide isostere **3** is 55%. The major impurity results from competitive *O*-alkylation.[13] Thirdly, significant epimerization at the existing stereogenic center C-5 occurred under a variety of chemical conditions to remove the ethyl protecting group in **3**. The difficulty in removing the minor wrong enantiomer makes the elongation from the C-terminal problematic. Consequently, by using this protocol, the synthesis of **1** requires ten steps with an overall 10% only yield starting from 4-fluorobenzaldehyde.[11]

These issues promoted us to look for alternative routes and we describe herein a chemoenzymatic method for the synthesis of tripeptide isostere **1**. The method is short with high yields and excellent enantiomeric excesses.

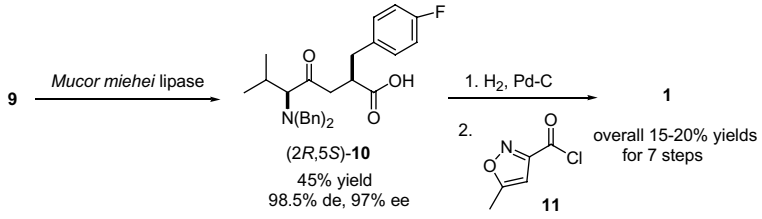
2. Results and discussion

The synthesis starts with *O*-benzyl (*S*)-valine ester **5**, which can be readily prepared from (*S*)-valine **4** in good yields (Scheme 2),[14] followed by acyl transfer to the carbanion of a methyl phosphonate to give β-ketophosphonate **6**. Subsequently, under Horner–Emmons conditions, this compound was treated with NaH in THF and reacted with α-ketoester **7**, which was prepared in one pot from 4-fluorobenzyl chloride in good yields (85%).[15,16] The resulting α,β-unsaturated γ-ketoester **8** was obtained as a mixture of *E*- and *Z*-isomers (5:1) with an overall 54% yield for three steps. Initial efforts were directed toward stereocontrolled reduction of this trisubstituted alkene to generate the desired stereogenic center at C-2 in **9** through either stereoinduction or chiral ligands. However, all of the tested conditions gave either low yields or poor stereoselectivity.[17] Finally standard hydrogenation using Pd/C was applied to produce a mixture of two diastereomers (2*R*,5*S*)- and (2*S*,5*S*)-**9** in good yields (95%). It should be noted that all attempts to remove the ester group in **9** by chemical hydrolysis led to extensive epimerization at the stereogenic center C-5.

At this stage, enzymes were screened to catalytically resolve the diastereomeric mixture of **9** through ester hydrolysis (Scheme 3). The identification of appropriate enzymes relied on automated enzyme screening techniques reported recently in our lab.[18] A 96-well plate kit, including lipases, proteases, acylases, esterases, and amidases, was incubated with a diastereomeric mixture of **9** on a thermomixer for 4–6 h. The reactions were then quenched with acetonitrile and the results analyzed by HPLC. A lipase from the fungus *Mucor meihei* was identified as the best hit for a stereoselective hydrolysis of **9** to produce the desired acid (2*R*,5*S*)-**10**. It is worth noting that the enzyme is inexpensive thus allowing its use for large-scale production. Substrate concentration and pH were then examined for optimal conditions. It was found that **10** could be produced in good resolution yield (45%) and high enantiomeric excess (98.5% de, 97% ee, $E = 170$) at pH 7.5. Under these conditions, no epimerization at stereogenic center C-5 was identified in contrast to chemical hydrolysis. Finally, the target compound **1** was obtained in good yields (70–75%) and high enantiomeric excess (98% de, 97% ee) over two steps via hydrogenolysis of **10** followed by acylation with **11** under Schotten–Baumann conditions.[19] The desired stereochemistry at C-2 and C-5 was confirmed by comparing to the literature data.[7,8]



Scheme 2.



Scheme 3.

By this approach, the desired ketomethylene tripeptide isostere **1** was synthesized in 15–20% yields overall, over seven steps starting from (*S*)-valine **4**. The process has shorter sequences and higher yields than the chiral alkylation route (15–20% vs 10%). The key step is an enzyme catalyzed ester hydrolysis, which produces the desired stereoisomer without epimerization of the existing stereogenic center at C-5.

3. Conclusion

In summary, a chemoenzymatic method has been developed for the synthesis of a ketomethylene tripeptide isostere. This process has higher yields and a shorter sequence than reported methods. This methodology can potentially be extended to the preparation of a variety of ketomethylene peptide isosteres with different substituents at C-2 since α-ketoesters can be readily prepared in one step with excellent yields (85–98%) from alkyl or aryl halides.[16] For example, branched substituents might be installed at C-2 with the desired stereochemistry, where poor diastereoselectivity would be expected using the chiral alkylation protocol.[10]

4. Experimental

The majority of enzymes utilized in the preparation of screening kits were obtained from various enzyme suppliers including Amano (Nagoya, Japan), Roche (Basel, Switzerland), Novo Nordisk (Bagsvaerd, Denmark), Altus Biologics Inc. (Cambridge, MA), Biocatalytics (Pasadena, CA), Toyobo (Osaka, Japan), Sigma–Aldrich (St. Louis, MO, USA). HPLC analysis of the screened samples was performed on an Agilent 220 HPLC autosampler. Reactions were performed in an Eppendorf thermomixer-R (VWR). Solvents used during optimization were obtained from EM Science (Gibbstown, NJ) and were of the highest purity available. Chiral HPLC columns used in analysis were obtained from Chiral Technologies (Exton, PA) and Phenomenex (Torrance, CA). Each HPLC sample was prepared by taking 5×200 μL from the reaction slurry and then combined and diluted with 4 mL of acetonitrile. 100 μL of that solution was further diluted with 400 μL of acetonitrile and injected into HPLC. For compound **1**, the following chiral HPLC method was used: Chiralcel OJ-R (4.6100 mm, 3 μm); flow rate 0.5 mL/min; injection volume 10 μL; mobile phases: (A) 25 mM NaH_2PO_4 pH 2.0; (B) acetonitrile; isocratic: 40% B for 31 min, detection at 254 nm. LC–MS conditions were as follows: ESI (positive and negative modes); LC chromatography runs on Luna C_{18} column (30×4.6 mm, 3 μm) using ACN/H_2O containing 0.1% formic acid as mobile phases; gradient 10–90% ACN in 3.5 min, then isocratic with 90% ACN for 1 min; flow rate 0.8 mL/min; detection at 254 nm.

4.1. General procedure for enzyme screening

A screening kit (see Ref. 18) was thawed for 5 min and 80 μL of potassium phosphate buffer (0.1 M, pH 7.2) then dispensed into each well via a multi-channel pipette. ACN solution (10 μL) of the substrate (10–20 mg of **9** per milliliter) was then added to each well, and the plate incubated at 30 °C on a thermomixer (900 rpm). The reactions were quenched with 100 μL of acetonitrile after 5 h. The 96-well plate was then centrifuged, and the supernatant transferred from each well into another 96-well plate and analyzed with automated HPLC using a short C_{18} column (30×4.6 mm, 3 μm) and ACN/H_2O containing 0.1% TFA as the solvent system.

4.2. Tribenzyl ester 5

This compound was prepared according to a literature protocol.[14] ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32 (m, 15H), 5.29 (d, $J = 12.3$ Hz, 1H), 5.15 (d, $J = 12.3$ Hz, 1H), 3.97 (d, $J = 14.1$ Hz, 2H), 3.29 (d, $J = 14.1$ Hz, 2H), 2.15 (m, 1H), 1.01 (d, $J = 6.3$, 3H), 0.76 (d, $J = 6.3$ Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.2, 140.0, 130.1, 129.0, 128.8, 128.0, 68.6, 65.6, 55.1, 26.7, 20.0, 16.4.

4.3. β-Ketophosphonate 6

To a solution of dimethyl methyl phosphonate (12.4 g, 100 mmol) in THF (80 mL) at −78 °C was added dropwise BuLi (2.5 M in hexanes, 40 mL, 100 mmol). After 30 min, tribenzyl ester **5** (6.5 g, 16.7 mmol) in THF (10 mL) was added and the mixture stirred for 20 min at the same temperature followed by 3 h at rt. The reaction was then quenched with satd NH_4Cl (200 mL), and extracted with MTBE (100 mL × 2). The combined organic layer was washed with brine (60 mL), dried over $MgSO_4$, and concentrated to afford **6** as a crude product (5.6 g, 13.9 mmol, 85% in yields, 90–95% UV purity), which was carried over to the next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.28 (m, 10H), 2.80–4.2 (sets of m, 13H), 2.33 (m, 1H), 1.14 (d, $J = 6.0$ Hz, 3H), 0.80 (d, $J = 6.0$ Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 202.2, 139.5, 132.8, 129.0, 127.3, 71.1, 54.5, 52.8, 41.8, 40.1, 26.9, 19.0. ESI *m/z* $[M+H]^+$ 404.2.

4.4. β-Ketoester 7

This compound was prepared according to a literature protocol with consistent NMR data.[15,16] IR (KBr) 3421, 2974, 1725, 1504, 1221 cm^{-1}. FABMS *m/z* $[M+H]^+$ 211.

4.5. γ-Ketoester 9

To a stirred solution of NaH (60% in mineral oil, 0.7 g, 17.6 mmol) at 0 °C was added slowly a solution of **6** (6.5 g, 16.0 mmol) in THF (80 mL). A solution of **7** (3.7 g, 17.6 mmol) in THF (15 mL) at the same temperature was subsequently added. The ice bath was then removed and the reaction kept at rt. After 18 h, the reaction was quenched by water (50 mL) and extracted with MTBE (50 mL × 2). The combined organic layer was washed with brine (50 mL), dried over $MgSO_4$, and concentrated to afford **8** (5.5 g, 75%) as a crude

red oil. Without further purification, the crude **8** (4 g, about 8.2 mmol) was dissolved in ethanol (40 mL), and the mixture hydrogenated in the presence of 10% Pd/C at 1 atm for 5 h. The reaction mixture was filtered through Celite, which was washed with ethanol (10 mL) and the combined organic layer concentrated to give **9** (5.8 g, 12 mmol, ca. 75% over two steps from **6**) upon chromatographic purification (hexanes/ethyl acetate 95:5) over three steps as a mixture of two diastereomers (1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.17–6.87 (m, 14H) 4.10–3.96 (m, 2H), 3.86 (dd, J = 6.0, 9.0 Hz, 2H), 3.37 (d, J = 14.4 Hz, 1H), 3.22 (d, J = 14.4 Hz, 1H), 3.05 (m, 1H), 2.89 (d, J = 10.8 Hz, 1H), 2.92 (d, J = 10.5 Hz, 1H), 2.65–2.85 (m, 1H), 2.49–2.59 (m, 1H), 2.16–2.38 (m, 2H), 1.20 (m, 6H), 1.05 (m, 3H), 0.70 (d, J = 6.3 Hz, 3H), 0.60 (d, J = 6.3 Hz, 3H). ^{13}C NMR (75 ppm, $CDCl_3$): δ 210.93, 210.84, 174.94, 174.84, 140.11, 139.89, 134.76, 134.57, 130.89, 130.79, 129.09, 128.97, 128.73, 127.41, 115.93, 115.50, 70.88, 70.76, 61.06, 54.94, 54.79, 47.98, 47.45, 41.93, 41.64, 37.41, 37.36, 27.74, 27.64, 20.62, 20.39, 20.36, 14.41, 14.53.

4.6. (2*R*,5*S*)-Acid 10

To a 150 mL jacketed flask equipped with a pH electrode, ester **9** (3 g, 6.13 mmol) in heptane (12 mL) was added followed by *Mucor miehei* lipase (30 mL, Sigma brand) in 18 mL of phosphate buffer (pH 7.5, 0.5 M). The reaction mixture was stirred at 25 °C at pH 7.5, which was maintained by an auto pH titrator. The conversion and diastereoselectivity was monitored by HPLC. When the conversion approached 45% (ca. 48 h), the reaction mixture was adjusted to pH 4.5 and extracted with heptane (60 mL × 2). The heptane layer was combined and extracted with methanol (containing 0.5% water) and the methanolic phase washed with heptane. After removal of methanol, the residue was slurried in hexane to afford the desired acid **10** (1.3 g, 2.81 mmol, 45% in yield, de 98.5%, ee 97%, 96% purity). ^{1}H NMR (300 MHz, CD_3OD): δ 7.05–7.38 (m, 14H), 3.36 (s, 2H), 3.27 (d, J = 13.8 Hz, 1H), 3.05 (m, 2H), 2.92 (d, J = 10.5 Hz, 1H), 2.54–2.66 (m, 2H), 2.40 (dd, J = 3.3, 18.9 Hz, 1H), 2.15 (m, 1H), 1.19 (m, 1H), 1.05 (d, J = 6.6 Hz, 3H), 0.74 (d, J = 6.3 Hz, 3H). ^{13}C NMR (75 ppm, CD_3OD): δ 210.78, 178.50, 139.81, 135.75, 130.80, 128.81, 128.28, 127.05, 115.26, 114.98, 70.08, 63.81, 63.16, 54.53, 42.85, 37.16, 27.34, 19.80, 19.44. ESI $[M+H]^+$ *m*/*z*: 462.2.

4.7. Tripeptide isostere 1

Compound **10** (680 mg, about 1.06 mmol) was dissolved into EtOH (10 mL) followed by H_2SO_4 (116 mg, 1.10 mmol) and 10% Pd–C (204 mg) in a Parc-shaker (50 psi). After about 5 h, the reaction finished as indicated by HPLC and TLC, and the mixture was then dissolved into methanol (75 mL), filtered through a short pad of Celite, which was then washed with another aliquot of methanol (75 mL). The combined solvent was evaporated to give a crude colorless oil (490 mg), without further purification, which was then dissolved into dioxane (40 mL) and the mixture then cooled to 10 °C. To the solution was added slowly DIPEA (0.45 mL, 2.60 mmol) in dioxane (5 mL), followed by slow addition of an isoxazole acid chloride **11** (153 mg, 1.06 mmol) in dioxane (10 mL). After 2 h at the same temperature, the reaction was finished as indicated by HPLC and TLC (methylene chloride/hexanes/IPA = 79/20/3) and to this mixture added methylene chloride (20 mL). The organic layer was separated, washed with 1 M HCl (10 mL), satd $NaHCO_3$ (10 mL), dried over $MgSO_4$, and filtered through a short pad of silica gel to give **1** (216 mg, 0.56 mmol, about 72%, 98% de, 97% ee) upon chromatographic purification (methylene chloride/hexane/IPA = 79/20/3). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.31 (d, J = 9.0 Hz, 1H), 7.14 (dd, J = 5.6, 8.5 Hz, 2H), 6.99 (t, J = 9.0 Hz, 2H), 6.40 (s, 1H), 4.70 (dd, J = 4.3, 8.6 Hz, 1H), 3.20 (m, 1H), 3.07 (dd, J = 6.0, 13.7 Hz, 1H), 2.98 (t, J = 9.4 Hz, 1H), 2.76 (dd, J = 8.3, 13.6 Hz, 1H), 2.56 (dd, J = 4.4, 18.3 Hz, 1H), 2.48 (s, 3H), 2.32 (m, 1H), 1.02 (d, J = 6.8 Hz, 3H), 0.85 (d, J = 6.8 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 206.28, 179.19, 171.73, 159.83, 158.57, 133.98, 130.86, 116.01, 115.73, 101.75, 62.77, 41.77, 36.91, 30.64, 20.27, 17.04, 12.61. ESI $[M-H]^-$ *m*/*z*: 389.2.

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13. The best ratio of *C*- over *O*-alkylation was found to be 3:1. The reaction is highly dependent upon solvents and bases. For example, using NaH, the reaction is complete within 16–24 in THF (3:1 *C*-/*O*-alkylation), while the use of *t*-BuOK or Cs_2CO_3 leads to the consumption of starting materials within 1–5 h (2:1 *C*-/*O*-alkylation). The alkylation takes place faster in more polar solvents than less polar ones. For example, no products were detected in toluene and methylene chloride at rt after 24 h.[11]
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17. For example, poor activities were identified for Crabtree's, Noyori's ligands, Wilkinson's catalyst, catecholborane, catecholborane/$(PPh_3)_3RhCl$, $LiAlH_4$/CuI, Zn/AcOH, and Me_2CuLi. Sodium borohydride mostly gave the product resulting from the ketone reduction, while Stryker's reagent led to poor stereoselectivity.[11]
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