Synthesis of nNOS-Capon interaction inhibitors: ZLc-002 and its derivatives

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Abstract

nNOS-Capon interaction is involved in anxiety disorder, disrupting the nNOS-Capon interaction has been demonstrated to show anxiolytic-like effect. In this protocol, N-(2-carbomethoxyacetyl)-D-valine methyl ester, a nNOS-Capon interaction inhibitor, we named it ZLc-002, was prepared by condensation of D-valine methyl ester hydrochloride with methyl malonyl chloride. The synthesis of some analogues of ZLc-002 was also described.

Introduction

Anxiety disorders are highly prevalent psychiatric diseases[1]. Selective serotonin reuptake inhibitors (SSRIs) and benzodiazepines (BZDs) are the most commonly prescribed anxiolytics. However, severe side effects for BZDs and taking effect much slowly for SSRIs render their use problematic[2]. Thus, there is need to develop novel anxiolytic agents that have quicker anxiolytic potential and are clinically well tolerated.

Several lines of evidence suggest that nNOS plays a pivotal role in the pathogenesis of anxiety disorders[3]. nNOS contains a PDZ domain that can interact with a variety of other proteins including post-synaptic density protein 95 (PSD-95) and carboxy-terminal PDZ ligand of nNOS (CAPON)[4], a scaffolding protein that regulates dendrite and synapse, and is associated with increased severity of posttraumatic stress disorder and depression[5]. The N-terminal phosphotyrosine binding domain of CAPON binds to dexamethasone-induced ras protein 1 (Dexras1). Dexras1 is activated by S-nitrosylation induced by nNOS in response to N-methyl-D-aspartate receptors (NMDARs) stimulation in brain. It has been demonstrated that nNOS, Capon, and Dexras1 can form a ternary complex of nNOS-Capon-Dexras1. The nNOS–CAPON association facilitates nNOS activation of Dexras1[6]. In the brain, Dexras1 negatively regulates the phosphorylation of extracellular signal-regulated kinase (ERK)[7], a kinase substantially implicated in emotional behaviors. In a recent publication, we demonstrated that disruption of the interaction of nNOS and Capon exhibits significantly anxiolytic-like effect, furthermore, based on the structural feature of the nNOS PDZ domain that bonds to the C-terminal of Capon, a series of small molecular inhibitors of the PDZ domain were designed and synthesized, and

especially, the N-(2-carbomethoxyacetyl)-D-valine methyl ester, we named it ZLc-002, showed significant in vitro and in vivo anxiolytic activities[8]. In this protocol, the detailed synthetic procedures of the ZLc-002 and its analogues were described.

Reagents

N-Methylmorphinline (Sinopharm Chemical Reagent Co., Ltd (SCRC))

D-Valine methyl ester hydrochloride (GL Biochem Ltd)

Methyl malonyl chloride (SCRC)

Citric acid (SCRC)

Methyl succinyl chloride (SCRC)

L-Valine methyl ester (GL Biochem)

Methyl 3-bromopropionate (SCRC)

D-Phenylalanine methyl ester hydrochloride (GL Biochem)

Cyanoacetic acid (SCRC)

Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP; GL Biochem)

Triethylamine (Et_3N; SCRC)

N,N-Diisopropylethylamine (DIEA; SCRC)

Nitrobenzene (SCRC)

Methanol (CH_3OH; SCRC)

Dichloromethane (CH_2Cl_2; SCRC)

Ehtyl acetate (EtOAc; SCRC)

Diethyl ether (Et_2O; SCRC)

Sodium sulfate (Na_2SO_4; SCRC)

Sodium bicarbonate ($NaHCO_3$; SCRC)

Sodium chloride (NaCl; SCRC)

Sodium hydroxide (NaOH; SCRC)

Hydrochloride (HCl; SCRC)

Silica gel (200-300 mesh; Qingdao Haiyang Chem. Co., Ltd)

Potassium iodide (KI; SCRC)

Sodium azide (NaN_3; SCRC)

Equipment

Analytical balance (G&G Electronic, JJ124BC)

Heating and stirring mantles/heater

Water ring vacuum pump

Oil vacuum pump

Rotary evaporators

UV detector

Nuclear Magnetic Resonance (Bruker, AVANCE AV-300)

Agilent 6410B Triple Quadrupole LC/MS Electrospray System Bundle

Microwave apparatus

Procedure

Synthesis of *N*-(2-carbomethoxyacetyl)-D-valine methyl ester (ZLc-002) TIMING 20 h

1. Add *N*-methylmorphinline (2 ml, 18.45 mmol) dropwise to a solution of D-valine methyl ester hydrochloride (1.50 g, 9 mmol) in CH_2Cl_2 (35 ml) under -15°C.
2. Add methyl malonyl chloride (1 ml, 9.45 mmol) to the resulting reaction mixture while stirring for 30 min under -15°C. CAUTION Methyl malonyl chloride is acrid. Carry out this reaction in a well-ventilated fume hood. Since methyl malonyl chloride is highly sensitive to moisture, the reaction should be carried out under anhydrous conditions.
3. Allow the reaction to warm to room temperature and stir at room temperature for 12 h.
4. Remove the solvent under vacuum, dilute the residue with water (8 ml), and transfer

the solution into a 250-ml separation funnel. CAUTION ZLc-002 is water soluble, if too much water is added, it makes the extraction difficult and results a low yield.

5. Extract the solution with EtOAc (4 × 50 mL) and combine the organic phases.
6. Wash the solution with 10% citric acid solution (10 mL), 5% $NaHCO_3$ solution (10 mL), and saturated NaCl aqueous solution (2 × 50 mL) consecutively.
7. Dry the organic solution over anhydrous Na_2SO_4 for 0.5 h, filter the Na_2SO_4 using a fritted Buchner funnel into a 500-mL round-bottomed flask and concentrate under vacuum with a rotary evaporator.
8. Pack a chromatography column (2.8 × 40 cm) with a slurry of silica gel in EtOAc : PE = 1 : 2.
9. Transfer the crude product solution to the column with a Pasteur pipette. Wash the flask with 2 mL of solvent (EtOAc : PE = 1 : 2).
10. Elute the column with EtOAc : PE = 1 : 2.
11. Collect 25-mL fractions in test tubes. Identify the fractions containing the product using TLC (R_f = 0.5, EtOAc : PE = 1 : 2), and visualize it with iodine. Combine fractions which contain the product, and then remove the solvent under a rotary evaporator to yield the product as a crystalline solid.

Synthesis of *N*-(3-carbomethoxypropionyl)-D-valine methyl ester (ZLc-004) TIMING 20 h

1. Add Et_3N (3.42 mL, 24.6 mmol) dropwise to a solution of D-valine methyl ester hydrochloride (2 g, 12 mmol) in CH_2Cl_2 (40 ml) under -15°C.
2. Add methyl succinyl chloride (1.55 ml, 12.6 mmol) to the resulting reaction mixture while stirring for 30 min under -15°C. CAUTION Methyl succinyl chloride is acrid. Carry out this reaction in a well-ventilated fume hood. Since methyl succinyl chloride

is highly sensitive to moisture, the reaction should be carried out under anhydrous conditions.

3. Allow the reaction to warm to room temperature and stir at room temperature for 12 h.
4. Remove the solvent under vacuum, dilute the residue with water (20 ml), and transfer the solution into a 250-ml separation funnel.
5. Extract the solution with EtOAc (4 × 60 mL) and combine the organic phases.
6. Wash the solution with 10% citric acid solution (2 × 20 mL), 5% $NaHCO_3$ solution (2 × 20 mL), and saturated NaCl aqueous solution (2 × 50 mL) consecutively.
7. Dry the organic solution over anhydrous Na_2SO_4 for 0.5 h, filter the Na_2SO_4 using a fritted Buchner funnel into a 500-mL round-bottomed flask and concentrate under vacuum with a rotary evaporator.
8. Pack a chromatography column (2.8 × 40 cm) with a slurry of silica gel in EtOAc : PE = 2 : 3.
9. Transfer the crude product solution to the column with a Pasteur pipette. Wash the flask with 2 mL of solvent (EtOAc : PE = 2 : 3).
10. Elute the column with EtOAc : PE = 2 : 3.
11. Collect 25-mL fractions in test tubes. Identify the fractions containing the product using TLC (R_f = 0.65, EtOAc : PE = 1 : 2), and visualize it with iodine.
12. Combine fractions which contain the product, and then remove the solvent under a rotary evaporator to yield the product as a yellowish solid.

Synthesis of *N*-(2-carboxyacetyl)-D-valine methyl ester (ZLc-002-1) TIMING 15 h

1. Weigh out 1.92 g of *N*-(2-carbomethoxyacetyl)-D-valine methyl ester (ZLc-002, 8.3 mmol) in a 100 mL round-bottomed flask, add 9 ml of methanol.

2. Cool down to about 0℃ with a ice-water bath.
3. Add 9.15 mL of NaOH solution (1 mol/l, 9.15 mmol), and stir at 0℃ for 15 min.
4. Remove the ice-water bath and allow the reaction to stir at room temperature for 6 h.
5. Remove the methanol under reduced pressure.
6. Adjust the pH to 2~3 by using concentrated HCl solution.
7. Extract the residue with EtOAc (4 × 50 mL) and combine the extracts.
8. Wash the solution with saturated NaCl aqueous solution (2 × 50 mL).
9. Dry the organic solution over anhydrous Na_2SO_4 for 0.5 h, filter the Na_2SO_4 using a fritted Buchner funnel into a 500-mL round-bottomed flask and concentrate under vacuum with a rotatory evaporator.
10. Pack a chromatography column (2.8 × 40 cm) with a slurry of silica gel in EtOAc : PE : HOAc = 15 : 5 : 1.
11. Transfer the crude product solution to the column with a Pasteur pipette. Wash the flask with 2 mL of solvent (EtOAc : PE : HOAc = 15 : 5 : 1).
12. Elute the column with EtOAc : PE : HOAc = 15 : 5 : 1.
13. Collect 25-mL fractions in test tubes. Identify the fractions containing the product using TLC (R_f = 0.45, EtOAc : PE : HOAc = 15 : 5 : 1), and visualize it with bromophenol blue.
14. Combine fractions which contain the product, and then remove the solvent under a rotary evaporator to yield the product as a yellowish liquid.

Synthesis of *N*-(2-carboxyacetyl)-D-valine (ZLc-002-2) TIMING 15 h

1. Weigh out 1.16 g of *N*-(2-carbomethoxyacetyl)-D-valine methyl ester (ZLc-002, 5 mmol)) in a 100 mL round-bottomed flask, and add 10 ml of methanol.

2. Cool down to about 0℃ with an ice-water bath.
3. Add 11 mL of NaOH solution (1 mol/l, 11 mmol), and stir at 0℃ for 15 min.
4. Remove ice-water bath and allow the reaction to stir at 30℃ for 6 h.
5. Remove the methanol under reduced pressure.
6. Adjust the pH to 2~3 by using concentrated HCl solution.
7. Extract the residue with EtOAc (4× 50 mL) and combine the extracts.
8. Wash the solution with saturated NaCl aqueous solution (2 × 50 mL).
9. Dry the organic solution over anhydrous Na_2SO_4 for 0.5 h, filter the Na_2SO_4 using a fritted Buchner funnel into a 500-mL round-bottomed flask and concentrate under vacuum with a rotary evaporator.
10. Pack a chromatography column (2.8 × 40 cm) with a slurry of silica gel in EtOAc : PE : HOAc = 10 : 10 : 3.
11. Transfer the crude product solution to the column with a Pasteur pipette. Wash the flask with 2 mL of solvent (EtOAc : PE : HOAc = 10 : 10 : 3).
12. Elute the column with EtOAc : PE : HOAc = 10 : 10 : 3.
13. Collect 25-mL fractions in test tubes. Identify the fractions containing the product using TLC (R_f = 0.35, EtOAc : PE : HOAc = 10 : 10 : 3), and visualize it with bromophenol blue.
14. Combine fractions which contain the product, and then remove the solvent under a rotary evaporator to yield the product as a yellowish liquid.

Synthesis of *N*-(2-carbomethoxyacetyl)-L-valine methyl ester (ZLc-034) TIMING 20 h

☐

This compound was synthesized by the method described for ZLc-002.

Synthesis of *N*-(2-carbomethoxyethyl)-D-valine methyl ester (ZLc-006) TIMING 18 h

☐

1. Add triethylamine (3.24 mL, 24.6 mmol) to a stirring solution of D-valine methyl ester hydrochloride (2.0 g, 12 mmol) in methanol (50 ml).
2. Add methyl 3-bromopropionate (1.6 ml, 14.4 mmol) to the reaction solution.
3. Add small amount of potassium iodide (10 mg) as catalyst.
4. Stir for 20 min at room temperature.
5. Heat the reaction mixture to refluxing while stirring for 12 h.
6. Cool down to room temperature.
7. Remove the solvent with a rotary evaporator.
8. Add ether (30 mL) to the residue to precipitate the produced triethylamine hydrogen bromide salt, wash the filtrate cake with ether (2 × 10 mL).
9. Remove the solvent with a rotary evaporator.
10. Pack a chromatography column (2.8 × 40 cm) with a slurry of silica gel in EtOAc : PE = 2 : 3.
11. Transfer the crude product solution to the column with a Pasteur pipette. Wash the flask with 2 mL of solvent (EtOAc : PE = 2 : 3).
12. Elute the column with EtOAc : PE = 2 : 3.
13. Collect 25-mL fractions in test tubes. Identify the fractions containing the product using TLC (R_f = 0.65, $CHCl_3$: CH_3OH : HOAc = 90 : 8 : 2), and visualize it with iodine.
14. Combine fractions which contain the product, and then remove the solvent under a rotary evaporator to yield the product as a reddish brown liquid.

Synthesis of *N*-(2-carbomethoxyacetyl)-D-phenylalanine methyl ester (ZLc-011) TIMING 20 h

This compound was synthesized by the method described for ZLc-002.

Synthesis of *N*-(2-cyanoacetyl)-D-valine methyl ester TIMING 22 h

1. Add cyanoacetic acid (2.7 g, 59 mmol) to a solution of D-valine methy ester hydrochloride (5 g, 56 mmol) in methanol (60 ml) under 0℃.
2. Add DIEA (12 mL, 124 mmol) and stir for 5 mim.
3. Add PyBOP (18.6 g, 67 mmol) while stir for 30 min at 0℃.
4. Remove the ice-water bath, and stir the reaction mixture at room temperature for 12 h.
5. Remove the solvent with a rotary evaporator.
6. Dilute the residue with EtOAc 120 mL.
7. Transfer the solution to a 500-mL separation funnel, wash the solution with 10% citric acid solution (30 mL × 2), 5% $NaHCO_3$ solution (30 mL × 2), and saturated NaCl aqueous solution (30 mL × 2) consecutively.
8. Dry the organic solution over anhydrous Na_2SO_4 for 0.5 h, filter the Na_2SO_4 using a fritted Buchner funnel into a 500-mL round-bottomed flask and concentrate under vacuum with a rotary evaporator.
9. Pack a chromatography column (2.8 × 40 cm) with a slurry of silica gel in EtOAc : PE = 1 : 1.
10. Transfer the crude product solution to the column with a Pasteur pipette. Wash the flask with 2 mL of solvent (EtOAc : PE = 1:1).
11. Elute the column with EtOAc : PE = 1 : 1.
12. Collect 25-mL fractions in test tubes. Identify the fractions containing the product using TLC (R_f = 0.62, EtOAc : PE = 1 : 1), and visualize it with iodine.
13. Combine fractions which contain the product, and then remove the solvent under a rotary evaporator to yield the product as a white solid.

Synthesis of $_N$-(2-tetrazoylacetyl)-D-valine methyl ester (ZLc-008) TIMING 12 h

1. Add N-(2-cyanoacetyl)-D-valine methyl ester (4.0 g, 20 mmol) to nitrobenzene (30 mL).
2. Add sodium azide (1.69 g, 26 mmol) and triethylamine hydrochloride (3.56 g, 26 mmol) to the above solution.
3. Irradiate the reaction solution with a microwave apparatus at 100oC for 6 h.
4. Cool to room temperature, extract the solution with water (4 × 30 mL).
5. Combine the water extracts, wash the solution with ethyl ether (2 × 30 mL).
6. Acidify the solution with hydrochloride solution (2 M) to pH 2~3.
7. Extract the product with ethyl acetate (4 × 30 mL).
8. Combine the ethyl acetate extracts and wash the solution with saturated NaCl solution (2 × 30 mL).
9. Dry the organic solution over anhydrous Na_2SO_4 for 0.5 h, filter the Na_2SO_4 using a fritted Buchner funnel into a 500-mL round-bottomed flask and concentrate under vacuum with a rotary evaporator to about 10 mL.
10. Cool the solution in an ice-water bath, add petroleum ether (30 mL) to precipitate the product.
11. Collect the crystals by filtration, wash with small amount of petroleum ether and dry the product in a desiccator under vacuum for 2 h.

Anticipated Results

N-(2-Carbomethoxyacetyl)-D-valine methyl ester (ZLc-002)

Yield 50%, white crystal.

TLC (EtOAc : PE= 1 : 2): R_f = 0.5.

^{1}H NMR (300 MHz□$CDCl_3$) δ (ppm) : 0.96 (t, 6H, *J* = 6.48 Hz), 2.20 (s, 1H), 3.37 (s, 2H), 3.75 (s, 3H), 3.77 (s, 3H), 4.56 (s, 1H), 7.52 (s, 1H).

Mass (ESI) (m/z) $[M+H]^+$ calculated for $C_{10}H_{18}NO_5$, 232.25; found, 232.12.

N-(3-Carbomethoxypropionyl)-D-valine methyl ester (ZLc-004)

Yield 69%, yellowish solid.

TLC (EtOAc : PE = 2 : 3): R_f = 0.65.

^{1}H NMR (300 MHz，$CDCl_3$) δ (ppm) : 0.91 (d, 3H, *J* = 6.84 Hz), 0.94 (d, 3H, *J* = 6.81 Hz), 2.09～2.21 (m, 1H), 2.53～2.59 (m, 2H), 2.61～2.73 (m, 2H), 3.69 (s, 3H), 3.74 (s, 3H), 4.53～4.58 (q, 1H), 6.13～6.16 (d, 1H, *J* = 8.22 Hz).

Mass (ESI) (m/z) $[M+H]^+$ calculated for $C_{11}H_{20}NO_5$, 246.27; found, 246.10.

N-(2-Carboxyacetyl)-D-valine methyl ester (ZLc-002-1)

Yield 62.7%, yellowish liquid.

TLC (EtOAc : PE : HOAc = 15 : 5 : 1): R_f = 0.45.

^{1}H NMR (500 MHz，$CDCl_3$) δ (ppm) : 0.94 (d, 3H, *J* = 6.90 Hz), 0.96 (d, 3H *J* = 6.85 Hz), 2.19～2.24(m, 1H), 3.42 (s, 2H), 3.77 (s, 3H), 4.58～4.61 (q, 1H), 7.11 (s, 1H).

Mass (ESI) (m/z) $[M+H]^+$ calculated for $C_{10}H_{18}NO_5$, 232.25; found, 232.20.

N-(2-Carboxyacetyl)-D-valine (ZLc-002-2)

Yield 57.1%, yellowish solid.

TLC (EtOAc : PE : HOAc = 15 : 5 : 1): R_f = 0.35.

^{1}H NMR (500 MHz，DMSO-d_6) δ (ppm) : 0.88 (d, 3H, *J* = 3.20 Hz), 0.89 (d, 3H, *J* = 3.25 Hz), 1.99～2.06 (m, 1H), 3.28～3.31 (m, 2H), 4.16～4.19 (m, 1H), 8.18 (d, 1H, *J* = 8.6 Hz), 12.50 (s, 2H).

Mass (ESI) (m/z) $[M+H]^+$ calculated for $C_8H_{14}NO_5$, 204.19; found, 204.22.

N-(2-Carbomethoxyacetyl)-L-valine methyl ester (ZLc-034)

Yield 37.7%, yellowish liquid.

TLC (EtOAc : PE = 1 : 2): R_f = 0.5.

^{1}H NMR (500 MHz，$CDCl_3$) δ (ppm): 0.94 (d, 3H, *J* = 6.90 Hz), 0.97 (d, 3H, *J* = 6.90 Hz), 2.18～2.24 (m, 1H), 3.38 (s, 2H), 3.75 (s, 3H), 3.77 (s, 3H), 4.55～4.57 (q, 1H), 7.53 (d, 1H, *J* = 6.85 Hz).

Mass (ESI) (m/z) $[M+H]^+$ calculated for $C_{10}H_{18}NO_5$, 232.25; found, 232.19.

N-(2-Carbomethoxyethyl)-D-valine methyl ester (ZLc-006)

Yield 90.6%, reddish brown liquid.

TLC ($CHCl_3$: CH_3OH : HOAc = 90 : 8 : 2): R_f = 0.65.

^1H NMR (500 MHz，$CDCl_3$) δ : 3.73 (s, 3H), 3.67 (s, 3H), 3.02 (d, 1H, *J* = 6.10 Hz), 2.97～2.93 (m, 1H), 2.75～2.70 (m, 1H), 2.52～2.47 (m, 2H), 2.11 (s,1H), 1.93 (q, 1H, *J* = 6.80 Hz), 0.99～0.92 (m, 6H).

Mass (ESI) (m/z) $[M+H]^+$ calculated for $C_{10}H_{20}NO_4$, 218.26; found, 218.20.

N-(2-Carbomethoxyacetyl)-D-phenylalanine methyl ester (ZLc-011)

Yield 74.5%, yellowish liquid.

TLC (EtOAc : PE = 1 : 1): R_f = 0.7.

^1H NMR (300 MHz，$CDCl_3$) δ : 7.38 (d, 1H, *J* = 6.42 Hz), 7.32～7.22 (m, 3H), 7.13 (d, 2H, *J* = 7.32 Hz), 4.87 (q, 1H, *J* = 6.27 Hz), 3.72 (s, 6H), 3.30 (s, 2H), 3.21～3.05 (m, 2H)。

Mass (ESI) (m/z) $[M+H]^+$ calculated for $C_{14}H_{18}NO_5$, 280.29; found, 280.33.

N-(2-Cyanoacetyl)-D-valine methyl ester

Yield 48.7%, white solid.

TLC (EtOAc : PE = 1 : 1): R_f = 0.62

Mass (ESI) (m/z) $[M+H]^+$ calculated for $C_9H_{15}N_2O_3$, 199.22; found, 199.23.

N-(2-Tetrazoylacetyl)-D-valine methyl ester (ZLc-008)

Yield 43.5%, white solid.

TLC ($CHCl_3$: CH_3OH : HOAc = 90 : 8 : 2): R_f = 0.22

^1H NMR (300 MHz，$CDCl_3$) δ : 6.60 (s, 1H), 4.55 (dd, 1H, J_1 = 8.61 Hz, J_2 = 4.89 Hz), 3.77 (s, 3H), 2.71 (s, 1H), 2.27～2.16 (m, 1H), 0.96 (dd, 6H, J_1 = 8.37 Hz, J_2 = 7.11 Hz)。

Mass (ESI) (m/z) $[M+H]^+$ calculated for $C_9H_{16}N_5O_3$, 242.25; found, 242.30.

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Acknowledgements

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Figures

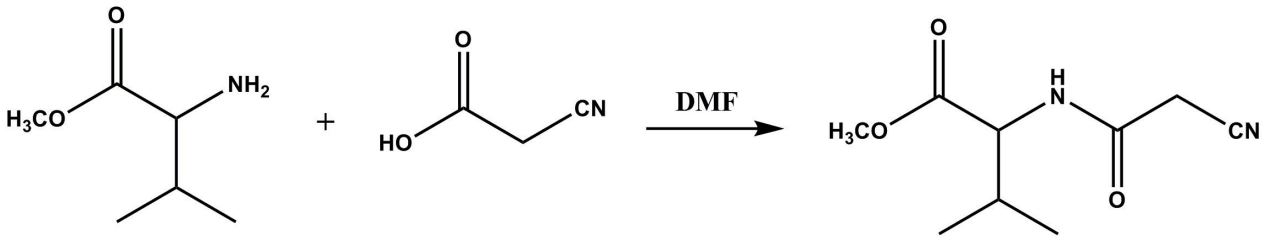

Figure 1

Figure 8 Synthesis of N-(2-cyanoacetyl)-D-valine methyl ester

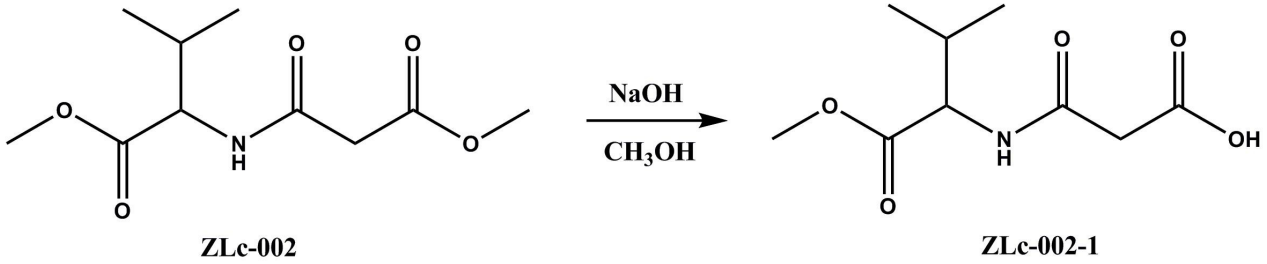

Figure 2

Figure 3 Synthesis of ZLc-002-1

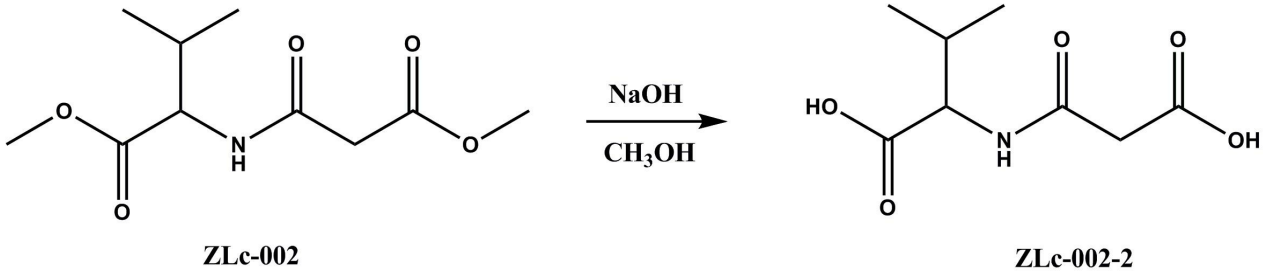

Figure 3

Figure 4 Synthesis of ZLc-002-2

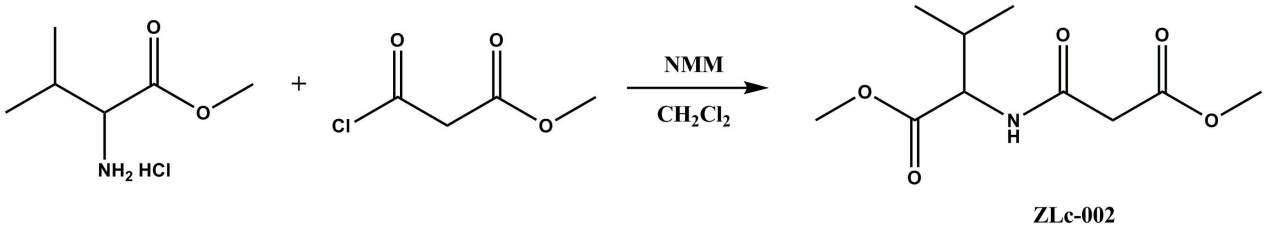

Figure 4

Figure 1 Synthesis of ZLc-002

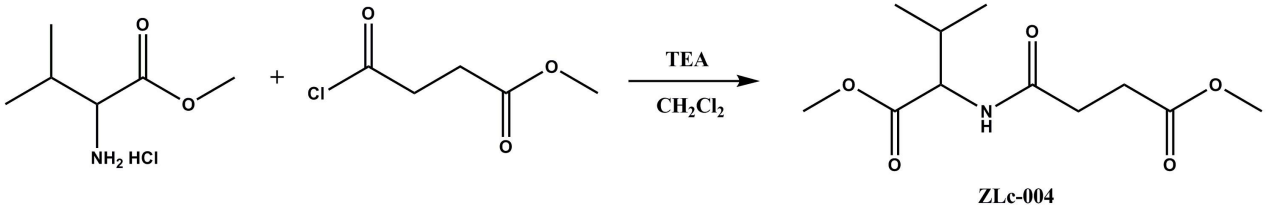

Figure 5

Figure 2 Synthesis of ZLc-004

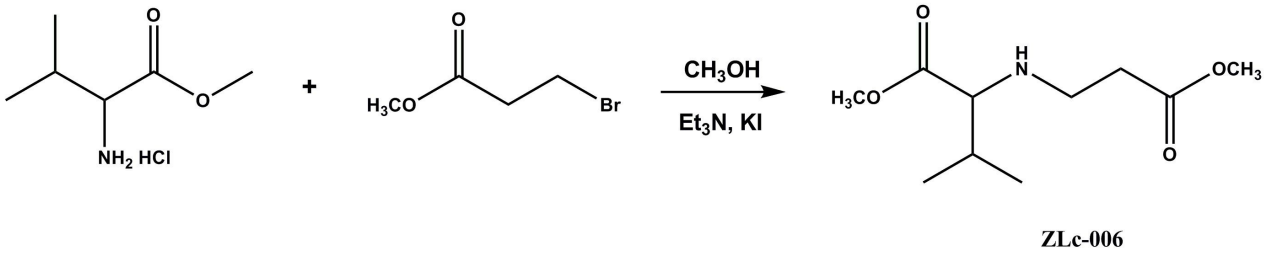

Figure 6

Synthesis of ZLc-006

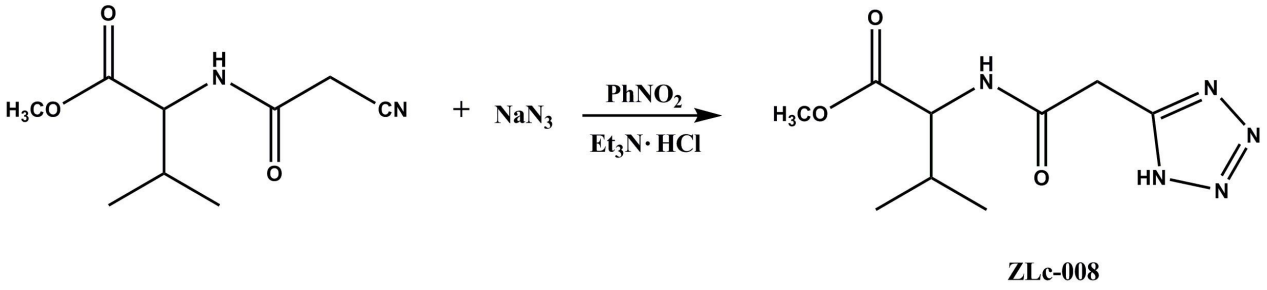

Figure 7

Figure 9 Synthesis of ZLc-008

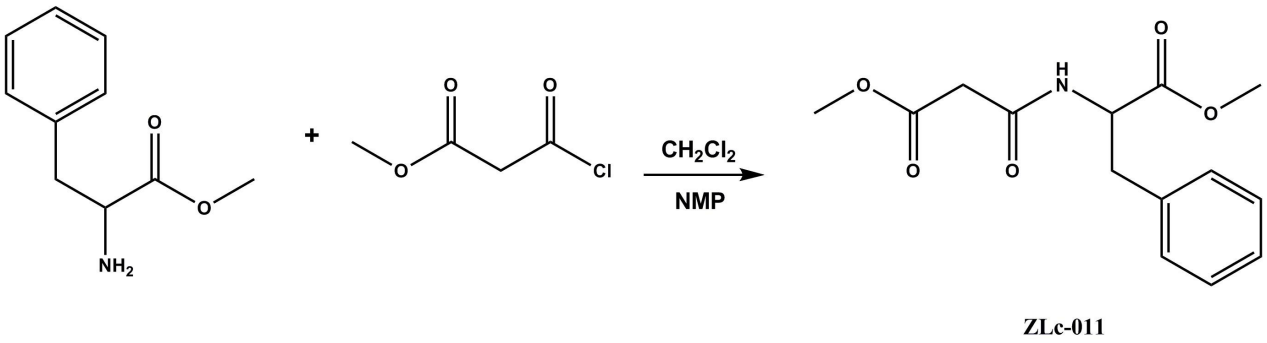

Figure 8

Figure 7 Synthesis of ZLc-011

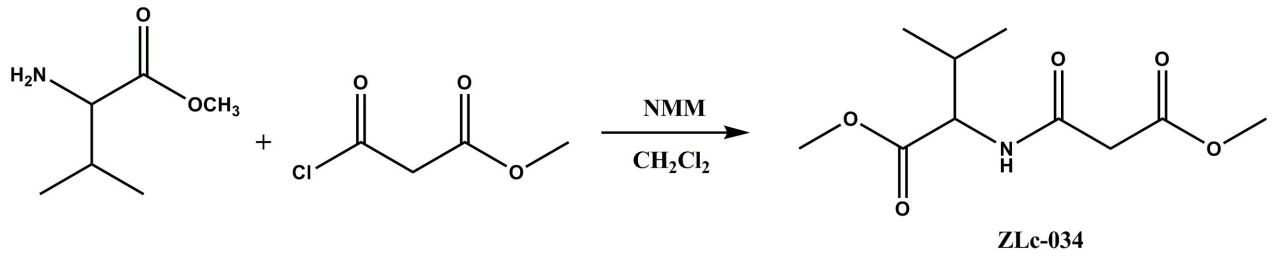

Figure 9

Figure 5 Synthesis of ZLc-034

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