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A Practical Method for Functionalized Peptide or Amide Bond Formation in Aqueous−Ethanol Media with EDC as Activator†

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Abstract:

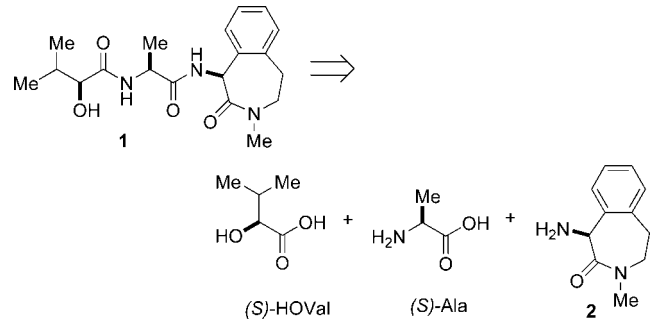
Selective formation of amides or peptides with ethanol as solvent using a convenient one-pot procedure in which acid activating agent *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) is simply added to a mixture of the carboxylic acid, amine, catalytic HOBt (1-hydroxy benzotriazole), and NMM (*N*-methylmorpholine) as base has been developed. Sensitive functionalities such as hydroxyl groups are well tolerated under the reaction conditions. In addition, isolation of products is usually by simple filtration from the reaction media. The scope and generality of this methodology was investigated.

Introduction

Peptide coupling reactions have significantly advanced in accord with the development of new coupling agents in organic synthesis.[1] The carboxamide group which occurs in many natural products, peptides, and pharmaceutical active compounds is an important functionality; therefore, methods for the synthesis of this important functional group with minimal loss of the optical integrity at the chiral center have been developed. Among these useful procedures commonly employed is condensation of carboxylic acids and amines via isolated or in situ activation of the carboxylic acid moiety. Activated forms of carboxylic acid for peptide coupling reactions are represented by acid halides,[2] anhydrides,[3] activated amides,[4] and esters.[5] Dicyclohexylcarbodiimide (DCC) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) as peptide coupling agents have particularly attracted organic chemists in their synthesis of complex molecules as the reagents enable acid activation in the presence of amines and, therefore, serve as convenient and useful agents.[6] However, to prevent racemization and obtain reasonable yield, additives such as HOBt are required in low dielectric constant solvent systems such as chloroform or methylene chloride.[7] Solvents with low dielectric constants increase the rate of formation and promote stability of the key activated EDC−carboxylic acid intermediate. Conversely, solvents possessing high dielectric constants such as DMF decrease the rate of formation and stability of the key transient intermediate during the carbon−nitrogen bond-forming process.[8]

In the process chemistry development of **1**, the synthesis required two amide bond formations (eq 1). The first-generation amide-forming steps consisted of methylene chloride and equimolar quantities of EDC and HOBt. These conditions were superior to other methods investigated for the following two reasons: (1) Chiral integrity of each asymmetric center was retained. (2) No protection of the HOVal hydroxyl group was required during the peptide-forming reaction. Starting with these key findings, we sought for other parameters that would improve the synthesis process on multikilogram scale.[9] Herein is reported the developed method for peptide bond formation utilizing EDC, catalyst HOBt, and aqueous ethanol as the reaction solvent for the synthesis of **1**. This contribution entails the synthesis and a general utility of the process development.

Equation 1. Retrosynthesis of **1**



Peptide synthesis in aqueous media has been documented in the literature for synthesis of peptides and proteins and for immobilization of proteins. Low to moderate yields have been attained with water-soluble carbodiimides as the carboxylic acid activator.[10,11] Thus, the synthesis of **1** was attempted under

† This manuscript is dedicated to the memory of our colleague and good friend Chris Schmid.

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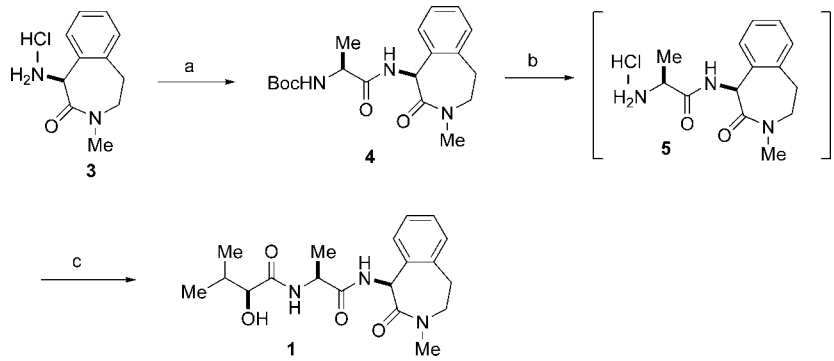
Scheme 1. **Synthesis of 4 and 1 using EDC and HOBt in water or ethanol**



similar conditions by coupling (*S*)-BocAla with **3** using EDC in water to obtain **4** and *in situ* cleavage of the Boc protecting group followed by coupling of (*S*)-HOVal with amine **5** in water to obtain **1** (Scheme 1). In this attempt, we were successful in obtaining **4** in 76% yield with excellent chiral purity (99.5%), but the coupling of (*S*)-HOVal to **5** was not successful. HOBt and HOAt (1-hydroxy-7-azabenzotriazole) are known to promote the peptide coupling and suppress racemization. We attempted the synthesis of **4** and **1** (Scheme 1) in the presence of stoichiometric amounts of HOBt using EDC. A solution of BocAla in water was obtained using *N*-methyl morpholine. To the solution was added one equivalent of EDC, one equivalent of HOBt, and one equivalent of **3**. After stirring at room temperature for 6 h, a 90% yield of **4** was isolated by filtration. Analysis of the crude product of **4** indicated that 2.5% HOBt was present as a byproduct.

The protecting Boc group in compound **4** was removed by using concentrated aqueous HCl, and the resulting product solution obtained (**5**) was used as such for the amide bond formation. (*S*)-HOVal, excess *N*-methyl morphiline, and equimolar quantities of EDC and HOBt were added to the solution of **5**. Product **1** was obtained in 85% yield with chiral purity (99.3%) after 16 h.

Although the two aqueous peptide couplings for the synthesis of **1** utilizing equimolar quantities of EDC and HOBt provided excellent yields and chiral purity, the slow reaction rate along with the crystallization process was of concern for pilot-plant processing. For example, as the reaction progressed, product **4** crystallized, and agitation of the solution became impeded due to uncontrolled crystallization. In order to achieve the desired outcome, the reaction had to be dilute to obtain desired yield and quality. Therefore, studies were undertaken to understand the impact of reagent addition order, type of bases and cosolvents using equimolar quantities of EDC and HOBt on the two peptide-forming reactions.

Based on the yield and quality data from the reaction parameters investigation, reagent addition order had no significant impact on either peptide reaction. Therefore, a single-pot procedure which involved the addition of EDC as the last reagent over time to a solution containing the carboxylic acid, amine coupling partner, HOBt, and base in water was investigated. The impacts of inorganic and organic bases in water or alcohol as a solvent were evaluated, mediated by EDC and HOBt. The data from both peptide reactions suggested that the peptide coupling was slow in water at room temperature when compared to the rate in alcohols as solvent. The coupled product was isolated in all cases in reasonable yield with excellent chiral purity. Under aqueous conditions, as the reaction progresses, the reaction became heterogeneous due to poor solubility of the product, and the reaction rate was slower. Alcohol reactions were homogeneous, and the reaction rate increased at 40 °C as compared to 22 °C. Water as solvent was higher in volume compared to ethanol (at least 6 times higher). The rate of reaction was at least 5 times faster compared to the the rate for reactions conducted in water. As a result of these observations, ethanol was chosen as the preferred reaction solvent. However, upon completion of the coupling reaction, water was introduced into the reaction mixture as an anti-solvent for the crystallization process. The product quality was excellent with respect to total related substances and chiral purity. An example of the reaction parameter investigation is presented in Table 1.

The primary use of additives such as HOBt in peptide coupling reactions is to enhance the reaction rate and suppress the racemization of the chiral center. However, examples of HOBt in peptide-forming reactions vary from catalytic to more than stoichiometric amounts. Therefore, we were interested in understanding the role of HOBt in the synthesis of **4** and **1** in ethanol as solvent. In the absence of HOBt, the coupling reactions progress slowly, providing a 76% yield of product after 38 h for **4**, whereas only an 11% yield of **1** was isolated after 24 h. Unreacted starting material was isolated in each case. On the basis of LC/MS data, the formation of *N*-acyl ureas was also observed in these "HOBt-free" reactions. Addition of catalytic amounts of HOBt ranging from 60% to 5% mol equiv increased the reaction rate. At 5% catalytic HOBt, >92% yield and >99% chiral purity was observed after only 7 h for both peptide-forming reactions in ethanol. More importantly, residual HOBt was not observed in the catalytic reactions. These catalytic observations of HOBt are similar to the work reported by Nozaki for the peptide coupling using EDC and HOBt or *N*-hydroxy-5-norbornene-2.3-dicarbodiimde in aqueous DMF.[12] An example of the catalytic performance of HOBt is presented in Table 2.

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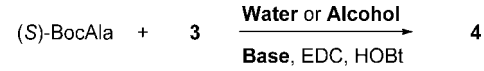
Table 1. **Investigation of coupling parameters in the synthesis of 4**



entry	solvent	base	reaction vol/time (h)	reaction *T* (°C)	yield (%)	chiral purity (%)
1	water	NMM	28/21	22	95	98.9
2	water	NMM	28/12	40	91	98.4
3	water	DIPA	28/48	22	92	97.9
4	water	5 N NaOH	28/69	22	94	98.9
5	water	5 N NaOH	28/30	40	90	98.5
6	water	5 N KOH	28 /66	22	85	98.9
7	EtOH	NMM	6/6	22	91	99.6
8	EtOH	NMM	6/3	40	90	99.7
9	EtOH	DIPA	6/6	22	90	98.6
10	MeOH	NMM	6 /3	40	80	98.7

Table 2. **Impact of HOBt stoichiometry on synthesis of 4 in ethanol**

entry	HOBt (mol %)	reaction time (h)	yield (%)	chiral purity(% ee)
1	0	38	76	99
2	5	7	92	99
3	15	6	92	99
4	30	5	91	100
5	45	4	91	100
6	60	3	89	100
7	100	3	90	98

Both aqueous and ethanol peptide coupling reactions met our goal of a reproducible process for pilot-plant execution. The optimized reaction consisted of ethanol as the reaction solvent. NMM was utilized as the base with 1.2 equiv of EDC, 5% M HOBt at 40 °C. Two of the major advantages of this process are the simple isolation of product by filtration and the need for no protection of the HOVal hydroxyl group. As a result, the overall synthesis was very cost-effective with short unit operation that times contributed to short cycle times.

Since EDC in aqueous or ethanol media is not a common methodology and such a coupling strategy was the only method that did not require protecting of the sensitive HOVal hydroxyl group, the scope and generality of the methodology was investigated. The emphasis of our investigation was on the utility of other molecules containing sensitive groups such as hydroxyls and on product isolation. Chart 1 summary consists of the methodology in the coupling of aliphatic, aromatic, α-hydroxy, *O*-hydroxy aromatic acids with (*S*)-α-methyl benzylamine and amino acids. In all cases amides and peptide bonds were formed in good to excellent yields. One common feature of these reactions was that product isolation was by simple filtration of the reaction mixture upon reaction completion. Further, the use of *tert*-butoxycarbonyl and carbobenzyloxy (Cbz) protected amino acids in the peptide formation was also accomplished. No epimerization of chiral center was observed in the products as indicated by ^{1}H NMR data. Also noted are the sensitive groups such as methyl sulfides, phenols, and aliphatic hydroxyls that tolerate the reaction conditions.

When compared to available peptide-forming methods, the EDC/HOBt approach offers several advantages. For example, the process is a simple one-pot procedure where reagents are combined and stirred at room temperature. Sensitive functional groups such as the hydroxyl group need no protection and are well tolerated. No potential competitive reactions such as ester formation was observed. Product isolation is usually by simple filtration. Hopefully, this methodology will offer an excellent alternative to the organic solvent-mediated peptide-forming reactions.[13]

General Methods. Reaction completion and product purity for all steps were evaluated by HPLC using the following RP-HPLC conditions: Zorbax C-8 column 25 cm × 4.6 mm, flow 1.0 mL/min; wavelength 220 nm; temperature 30 °C; injection volume: 20 μL of a ~0.05% solution in MeOH/water 1:1 v/v; eluent (A) MeOH, (B) (1 mL of H_3PO_4 in 1 L of H_2O; and gradient: (0 min) (A) 10%, (B) 90%; (20 min) (A) 85%, (B) 15%; (23 min) (A) 85%, (B) 15%; (24 min) (A) 10%, (B) 90%; (30 min) (A) 10%, (B) 90%. All reagents were commercially available except where indicated. Melting points were measured in open capillary tubes and are uncorrected. Optical rotations were obtained in methanol. ^{1}H NMR spectra were measured in DMSO-d_6 unless otherwise indicated, and IR spectra were taken using a KBr salt pellet.

Pilot-Plant Synthesis of 4 in Ethanol. To a reaction vessel was charged ethanol (358 L), **3**[14] (59.94 kg, 264.4 mol), HOBt (2.04 kg, 13.32 mol), (*S*)-BocAla (51.28 kg, 271.0 mol), and NMM (57.30 kg, 566.5 mol) to form a solution. EDC (56.58 kg, 295.1 mol) was then charged, and the reaction mixture was stirred at 25 °C for 10 h followed by the slow addition of water (541 L) over 1 h. The resulting slurry was filtered, rinsed with

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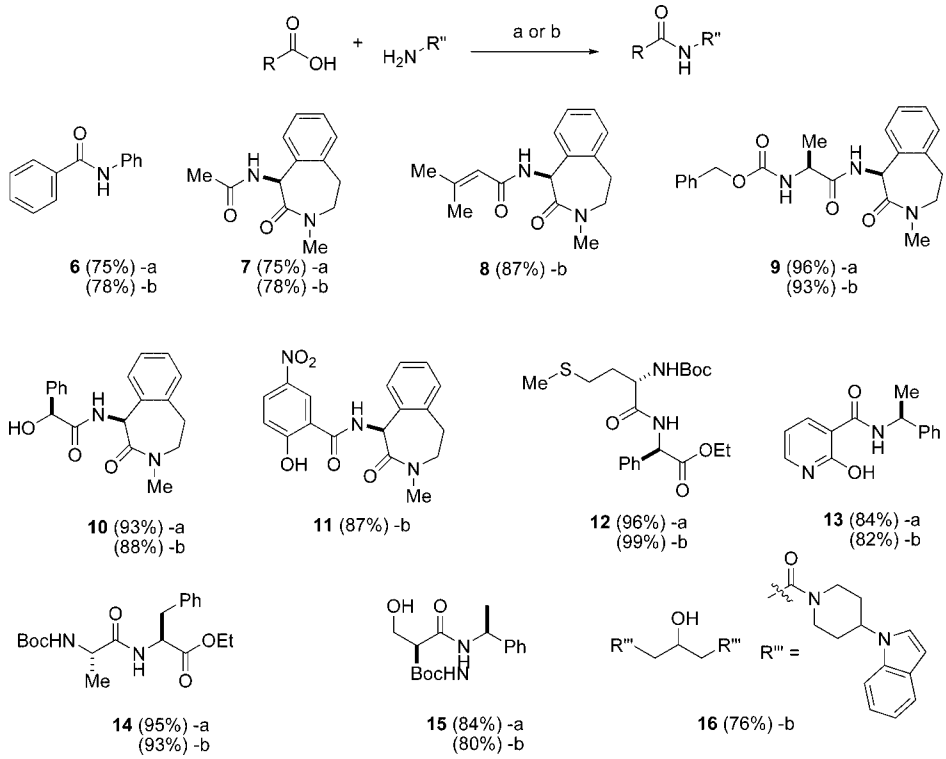
Chart 1. **Scope and generality of the EDC-mediated coupling methodology in aqueous EtOH**[a]



[a] Reagents and conditions: (a) Acid (1 equiv), amine (1 equiv), EDC (1 equiv), HOBt (1.15 M), water, 16−22 h, 22 °C. (b) EtOH was used in place of water during the reactions, and amount of HOBt was reduced to 5−15 mol %.

600 L of 80/20 water/EtOH, and dried to provide 88.155 kg of **4**; 91% yield; mp 215 °C; 99.98% ee. ^{1}H NMR (400 MHz) δ 1.25 (d, 3H, CH_3), 1.39 (s, 9H $C(CH_3)_3$), 2.9 (s, 3H, NCH_3), 3.23−3.46 (m, 3H, methylene protons), 4.13 (m, 2H, 2×CH), 6.12 (d, 1H, CH), 7.15−7.20 (m, 4 H, aromatic protons), 8.11 (d, NH); HRMS calcd for $C_{19}H_{27}N_3O_4$ 361.4447, found, 361.4442.

Pilot-Plant Synthesis of 1 in Ethanol. To a reaction vessel was charged ethanol (250 L), **4** (49.90 kg, 138.1 mol), and concentrated aqueous HCl (24.1 kg, 212.1 mol). The suspension was stirred and heated to 70 °C for 5 h. After cooling to 40 °C, NMM (23.8 kg, 235.3 mol) was added. At 0−5 °C, HOBt (2.11 kg, 13.78 mol) and water (7.58 kg) were added. The reaction mixture was then charged with hydroxyl valine (18.00 kg, 152.4 mol) followed by EDC (31.84 kg, 166.1 mol) and stirred at 5 °C for 1.5 h before heating to 40 °C for 3 h. Water (226 L) was slowly added over 1 h before seeding (0.8 kg in 3 L water). The resulting slurry was charged with water (502 L) over 2 h before cooling to 25 °C and then filtered. The cake was rinsed with water (317 L) and dried to afford 41.43 kg of **1**; 81.3% yield; mp 208 −212 °C; 99.5% ee; IR (cm^{-1}): 3323 (sh, O−H), 1687 and 1654 (sh, carbonyl stretching); ^{1}H NMR (400 MHz) δ 8.43 (d, 1H), 7.92 (d, 1H), 7.19 (m, 4H), 6.22 (d, 1H), 5.46 (br, 1H), 4.62 (t, 1H), 4.21 (m, 1H), 3.71 (s, 1H), 3.23 (m, 6H), 2.90 (s, 3H), 2.00 (t, 1H), 1.30 (d, 3H), 0.81 (dd, 6H); HRMS calcd $C_{19}H_{28}N_3O_4$ 362.2080, found, 362.2074.

General Procedure for Amide Formation Using EDC and HOBt in H_2O. To a 250 mL three-neck round-bottom flask equipped with an overhead stirrer and thermometer was charged water (48 mL), amine (0.01 mol), HOBt (0.01 mol), and the carboxylic acid (0.01 mol). NMM (0.037 mol) was added, and the reaction mixture stirred for 10 min and then cooled to 5 °C. EDC (0.011 mol) was added and the temperature allowed to rise to 25 °C. After stirring for 21 h, the mixture was filtered to collect the product and then dried.

General Procedure for the Amides Using EDC and Catalyst HOBt in Ethanol. To a 250 mL three-neck round-bottom flask equipped with mechanical stirrer was added the amine (1 mol), ethanol (6 vol), NMM (2.2 mol), carboxylic acid (1.03 mol), and HOBt (0.15 mol). The contents were then cooled to 10 °C, and EDC (1.2 mol) was added. The reaction was stirred at 25 °C for 3 h followed by the addition of water (10−18 vol). If the product is crystalline, the reaction mixture is filtered to collect the product. Noncrystalline products were isolated via extractive workup with EtOAc or methylene chloride as solvent.

6: method b: 78% yield; mp 160−163 °C; [Lit.[15] mp 160−163 °C].

7: method b: 78%; mp 234−237 °C; 99.3% ee; IR (cm^{-1}): 3335 (sharp N−H stretching), 1680 and 1640 (sharp amide C═O stretching); ^{1}H NMR (400 MHz) δ 2.04 (s, 3H, $COCH_3$), 2.90 (s, 3H, NCH_3), 3.2−3.75 (m, 3H, 3×CH), 4.12 (m, 1H, CH), 6.17 (d, 1H, CH), 7.12 (m, 4H, aromatic protons), 8.22 (d, 1H, CONH); HRMS calcd for $C_{13}H_{16}N_2O_2$ 232.2847, found, 232.2841.

8: method b: product was extracted in CH_2Cl_2 and concentrated to afford **8**; 87% yield; mp 198−207 °C; 98.9% ee; IR (cm^{-1}): 3319 (broad N−H stretching), 1680 (sharp C═O stretching), 1635 (C═O stretching); ^{1}H NMR ($CDCl_3$, 500

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MHz) δ 1.90 (s, 3H, CH_3), 2.19 (s, 3H, CH_3), 3.02 (s, 3H, $-NCH_3$), 3.20−3.36 (m, 3H, 3×CH), 4.29 (m, 1H, CH), 5.86 (1H, ═CH), 6.31 (d, 1H, CH), 6.91−7.61 (m, 5H, 4H, aromatic protons and −CONH); HRMS calcd for $C_{16}H_{20}N_2O_2$ 272.3509, found, 272.3497.

9: method b: 93% yield; mp 210−212 °C; 96.9% de; IR (cm^{-1}) 3315 and 3249 (sharp N−H stretching), 1709 (sharp C═O stretching), 1668 and 1658 (sharp amide C═O stretching); ^{1}H NMR (500 MHz) δ 1.3 (d, $CHCH_3$), 2.9 (S, NCH_3), 3.16−3.38 (m, 3H, −CH and $-CH_2$), 4.29 (m, 2H), 5.05 (s, CH_2), 6.1 (d, CH), 7.15−7.36 (m, 9H aromatic protons), 7.75 (d, NH), 8.22 (d, NH); HRMS calcd for $C_{22}H_{25}N_3O_4$ 395.4621, found, 395.4623.

10: method b: 88% yield; mp 170−174 °C; 98.7% de; IR (cm^{-1}): 3430 (broad O−H stretching), 3320 (broad N−H stretching), 1681 and 1641 (sharp carbonyl stretching); ^{1}H NMR (500 MHz) δ 2.91 (s, 3H, $-NCH_3$), 3.12−3.75 (m, 3H, 3×CH), 4.12 (m, 1H, CH), 5.17 (d, 1H, −OH), 6.12 (d, CH), 7.14−7.46 (m, 9H, aromatic protons), 8.33 (d, 1H, −CONH); HRMS calcd for $C_{19}H_{20}N_2O_3$ 324.3829, found, 324.3826.

11: method b: 87% yield; mp 195−197 °C; 98.7% ee; IR (cm^{-1}) 3400−3200 (broad, O−H and N−H stretching), 1662 and 1642 (sharp, amide carbonyl stretching); ^{1}H NMR (500 MHz) δ 2.93 (s, 3H, $-CH_3$), 3.2 −3.4 (m, 3H, 3×CH), 4.32 (m, 1H CH), 6.43 (d, 1H, CH), 7.09 −7.20 (m, 4H, aromatic protons), 7.73−7.81 (m, 2H, 2×CH aromatic protons), 8.13 (d, 1H, aromatic proton), 9.75 (d, NH); HRMS calcd for $C_{18}H_{17}N_3O_5$ 355.3533, found, 355.3531.

12: method b: 99% yield; mp 105−108 °C; $[\alpha]^{20}_D$ −98.51° (*c* 1.00, MeOH); IR (KBr cm^{-1}) 3320 (sharp N−H stretching), 1735 (sharp C═O stretching), 1652 (sharp amide C═O stretching); ^{1}H NMR (500 MHz) δ 1.35 (s, 9H, $-C(CH_3)_3$), 1.78 (m, 2H, CH_2), 1.9 (s, 3H, SCH_3), 2.36 (m, 2H, $-CH_2$), 3.61 (s, 3H,$COOH_3$), 4.10 (m, CH), 5.41 (d, CH), 7.01 (d, NH), 7.31 (m, 5 H aromatic protons), 8.56 (d, NH); HRMS calcd for $C_{19}H_{28}N_2O_5S$ 396.5094, found, 396.5091.

13: method b: 82% yield; mp 193−195 °C; $[\alpha]^{20}_D$ +127.19° (*c* 1.00, MeOH); IR (cm^{-1}) 3400−3200 (broad, O−H and N−H stretching), 1679 (sharp, amide carbonyl stretching); ^{1}H NMR (500 MHz) δ 1.44 (d, 3H, CH_3), 5.10 (m, 1H, CH), 6.47 (dd, 1H, aromatic proton), 7.23−7.34 (m, 5H, aromatic protons), 7.71 (d,1H, aromatic proton), 8.32 (1H, aromatic proton), 10.22 (1H, NH), 12.61 (broad OH); HRMS calcd for $C_{14}H_{14}N_2O_2$ 242.2799, found, 242.2795.

14: method b: product was isolated via extractive workup with EtOAc; 93% yield; mp 95−98 °C; $[\alpha]^{20}_D$ −23.62° (*c* 1.00, MeOH); IR (cm^{-1}) 3348 and 3330 (sharp N−H stretching), 1736 (sharp C═O strectching), 1679 (sharp amide C═O stretching); ^{1}H NMR (500 MHz) δ 1.7−1.12 (m, 6H, $-CHCH_3$ and $-CH_2CH_3$), 1.35 (s, 9H, $C(CH_3)_3$), 2.96 (m, 2H, CH_2), 4.01 (m, 3H, CH and $-OCH_2CH_3$), 4.85 (m, CH), 6.81 (d, NH), 7.21−7.25 (m, 5 H aromatic protons), 8.16 (d, NH); HRMS calcd for $C_{19}H_{28}N_2O_5$ 364.4454, found, 364.4451.

15: method b: 80% yield; mp 58 −61 °C; $[\alpha]^{20}_D$ −71.39° (*c* 1.00, MeOH); IR (cm^{-1}) 3323 (sharp O−H stretching), 3200 (broad N−H stretching), 1687 (sharp C═O strectching); ^{1}H NMR (DMSO-d_6) δ 1.32 (d, 3H, $CHCH_3$), 1.37 (s, 9H, $C(CH_3)_3$), 3.52 (m, 2H, CH_2), 3.97 (m, 1H, CH), 4.87 (broad, 1H, OH), 4.93 (q, 1H, −CH), 6.55 (d, NH), 7.15 −7.28 (m, 5H aromatic protons), 8.16 (d, NH); HRMS calcd for $C_{16}H_{24}N_2O_4$ 308.3807, found, 308.3804.

16: method b: 76% yield; mp 125 −126 °C; ^{1}H NMR (500 MHz) δ 1.81 (t, 4H), 1.96 (t, 4H), 2.57 (d, 4H), 2.71 (m, 2H), 3.26 (m, 2H), 4.09 (m, 2H), 4.6 (m, 2H), 4.69 (m, 2H), 6.42 (d, 2H, aromatic), 7.03 (t, 2H, aromatic), 7.10 (t, 2H, aromatic), 7.45 (d, 2H, aromatic), 7.52 (d, 2H, aromatic), 7.57 (d, 2H, aromatic); ^{13}C NMR (500 MHz) δ 40, 40.6, 44.7, 31.76, 32.52, 52.24, 65.4, 101.01, 109.79, 120.43, 124.92, 127.60, 135.22, 169.15; HRMS calcd for $C_{31}H_{36}N_4O_3$ 512.6576, found, 512.6571.

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