Pyrrolidine analogs of arylceramide HPA-12 †

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Abstract: Conformational constraint is the usual way to modify the properties of bioactive molecules. In some cases, such modification improves their activity as well as their affinity for their biological target. At the beginning of the millennium, the (*R,R*)-HPA-12 was found to be the first antagonist of ceramide transporter CERT, which has been identified as a key factor for the ER-to-Golgi trafficking of ceramides. Ten years later, we have revised the stereochemistry of the most active HPA-12 diastereomer to (*R,S*)-diastereomer and developed synthesis of more potent alkyl substituted HPA-12 analogs. In this contribution, we would like to describe a straightforward approach to the enantiomerically pure constraint pyrrolidine analogs of both (*R,S*)-HPA-12 and (*R,R*)-HPA-12 starting from 3-substituted pyroglutamic acids easily accessible *via* our methodology. The results of the binding assays to the CERT protein will be discussed.

Keywords: HPA-12; CERT protein; pyroglutamic acid; CIAT; Mitsunobu inversion

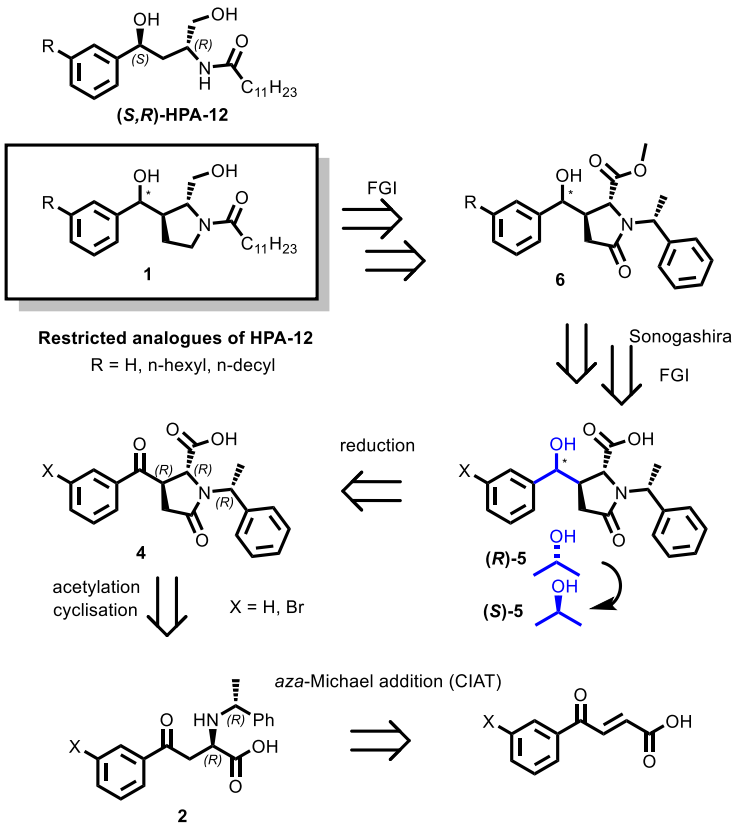
Introduction

Sphingolipids are membrane lipids that play important roles in the regulation of cell functions such as cell growth, differentiation, apoptosis, and homeostasis. Alterations in their metabolism have been associated with several pathologies. The regulation of these functions depends on a fine dynamic balance between some species whose relative intracellular concentrations are decisive for the cell´s fate. Ceramides are important signal molecules that control important cell vital functions such as division, apoptosis, or autophagy. They are formed in the endoplasmic reticulum and are the basis of glycosphingolipids and phospholipids whose synthesis takes place in the Golgi apparatus. Transport of ceramide from ER to GA provides CERT protein[1]. Aryl substituted ceramide analog - HPA-12 was discovered as the first specific inhibitor of intracellular transport of ceramides by CERT[2]. In this way, it is possible to regulate the ratio of sphingolipids and ceramides in the cell, which contributes to the proper functioning of the cell. The original structure of the HPA-12 was revised in 2011 by our research group[3] and finally confirmed by X-ray analysis[4]. In the last five years, several new synthetic methodologies of HPA-12 and analogs were published[5]. In our group, the substituted alkyl analogs of HPA-12 – structurally closer to the natural ligand of CERT protein – ceramide itself were prepared with m-hexyl HPA-12 analogs being the most effective ligand[6]. As part of our ongoing research focused on this field, we were particularly interested in the design and synthesis of constraint HPA-12 analogs starting from enantiomerically pure 3-aroyl pyroglutamic acids.

Methods

Scheme 1 illustrates the planned synthesis of the desired HPA-12 analogs (**1**). The design of the synthesis was based on the easy accessibility of enantiomerically pure γ-oxo-α-aminobutanoic acids (**2**) *via* own CIAT methodology[7,8]. The subsequent N-chloroacetylation followed by intramolecular cyclization represents a convenient and economical synthesis of substituted enantiomerically pure 3-

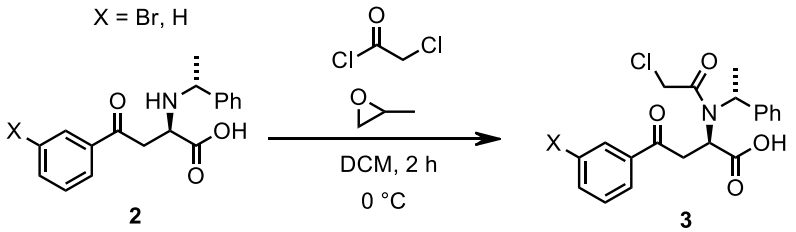
aroyl-5-oxo prolines (**4**). The important goal of their further elaboration was the stereoselective reduction of the carbonyl group. The main product in all tested conditions was the **(*R*)-5** stereoisomer. The desired **(*S*)-5** diastereoisomer was prepared by Mitsunobu inversion. The subsequent synthetic steps including standard functional groups manipulation, Sonogashira coupling, and several reduction steps followed our previous synthetic strategy published for alkyl analogs of HPA-12.



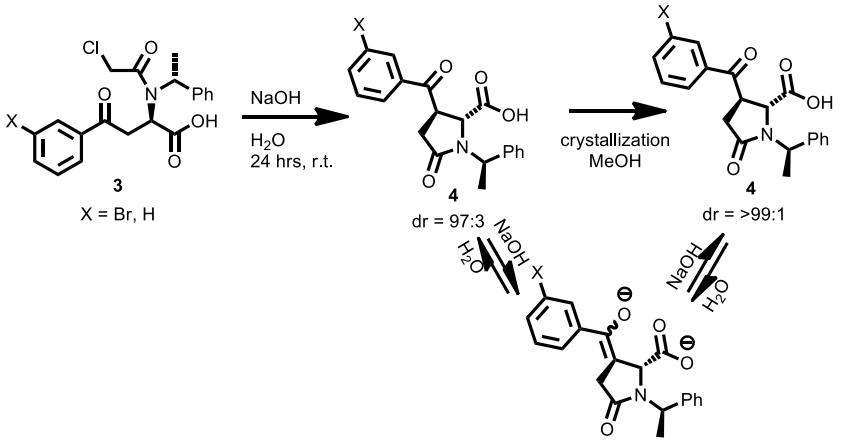
Scheme 1. Retrosynthetic analysis of the HPA-12 constrained analogs

The starting substituted oxoamino acids (**2**) were prepared using the *aza*-Michael addition of (*R*)-phenylethylamine to aroylacrylic acids in tandem with crystallization-induced asymmetric transformation (CIAT) [7]. This convenient multigram methodology was developed in our laboratory, and its main advantage is the simple product isolation by filtration.

The prepared oxo amino acids (**2**) were subsequently transformed into aroyl pyroglutamic acids (**4**) in two steps using N-chloroacetylation in non-basic condition with propylene oxide as an HCl scavenger[9]. The second step was highly stereoselective intramolecular cyclization using aqueous sodium hydroxide. The cyclisation provided almost pure diastereomer in high yield. The product is thermodynamically more stable *trans*-diastereomer with high stereoselectivity (d.r. 97:3). The practically pure diastereomer was obtained via simple crystallization from methanol.

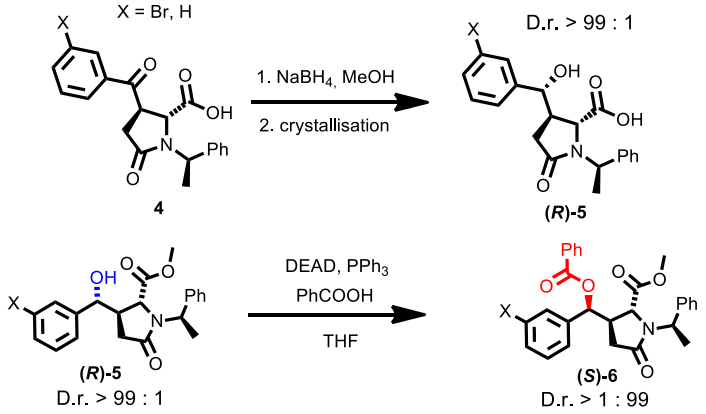


Scheme 2. Chloroacetylation of γ-oxo-α-amino acid



Scheme 3. Stereoselective cyclisation

The key transformation in our synthetic pathway to the targeted analogs of HPA-12 was the stereoselective reduction of carbonyl function. Despite testing a wide variety of methods for reducing a carbonyl group, we failed to prepare diastereomer with the correct **(*S*)-5** configuration. We decided to prepare him via Mitsunobu reaction by inversion of the major **(*R*)-5** diastereoisomer. The carboxyl function was protected by esterification with methyl iodide. The product can be purified by simple crystallization.



Scheme 4. Preparation of both stereoisomers of substituted pyroglutamic acid

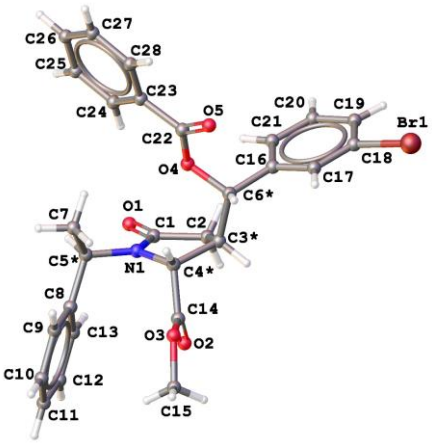
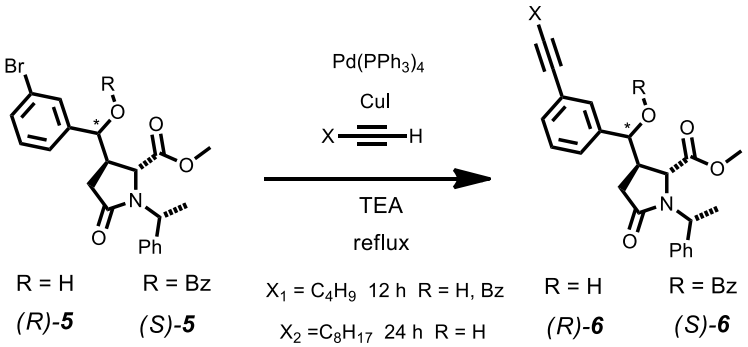


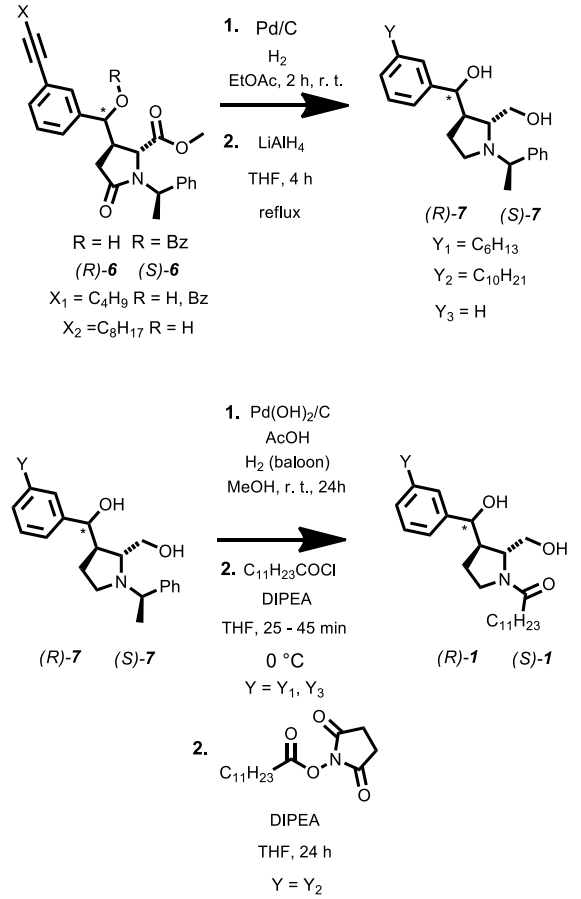
Figure 1. X-ray of the product of Mitsunobu inversion

The high affinity of m-hexyl-substituted HPA-12 for CERT protein led us to prepare also m-hexyl-substituted conformationally restricted pyrrolidine analogs of HPA-12. For this purpose, Sonogashira reaction was also used. Both prepared diastereomers with protected carboxyl function were applied as the starting materials for this reaction. Both reactions were carried out with high conversion.



Scheme 5. Sonogashira coupling

Final elaboration of polysubstituted oxoprolines to the targeted pyrrolidine analogs of HPA-12 was consistent with our previously published strategy.[6] For the preparation of the hexyl, substituent was used as a simple method of catalytic reduction of the unsaturated bond using palladium on carbon with hydrogen. Subsequent reduction by lithium aluminum hydride reduced the lactam and ester group to the desired prolinol derivative. The last step was catalytic N-debenzylation in the presence of two equivalents of acetic acid. The products were finally purified chromatographically. The final conformationally limited pyrrolidine analogs of HPA-12 were obtained by chemoselective N-acylation.



Scheme 6. Constrained HPA-12 analogs – final steps of synthesis

Results and discussion

This study describes conformationally restricted HPA-12 analogs with incorporated pyrrolidine scaffold as biologically active inhibitors of the CERT protein. The objectives of the work consisted of the preparation of aroyl-substituted oxoproline, reduction of the carbonyl group, preparation of the target pyrrolidine analogs of HPA-12 and determination of their chemical and physical characteristics. Research has been made to find out most effective synthetic methods for the preparation of optically pure secondary alcohols from the substituted ketones. Effective and simple reduction with sodium borohydride and crystallization resulted in the preparation of only one pure diastereomer with d.r. greater than 99: 1 in a yield of 60%. The opposite isomer was finally prepared using the Mitsunobu inversion. The inverse configuration at the newly-formed stereogenic center was confirmed by basic hydrolysis. Also, the ability of this product to well crystallize helped us by X-ray analysis to detect relative configurations of the resulting stereogenic center and thus to define the absolute configuration of the molecule. Further functional groups manipulation led to the synthesis of the final pyrrolidine analogs of HPA-12. Finally, both diastereomers of the target molecule were prepared and characterized by optical rotation measurement and HRMS spectra. Further optimization of the last synthetic steps is desirable, but the analogs have been prepared in sufficient quantity. Target pyrrolidine analogs of HPA-12 were sent for testing their affinity to the CERT protein using previously developed FRET methodology[10]. Preliminary results obtained from the Evotec International GmbH laboratory Toulouse, France show EC_{50} for CERT comparable with those for HPA-12.

Experimental section

Synthesis of m-Br HPA-12 analog, experimental procedures for key reactions:

Preparation of (R)-4-(3-bromophenyl)-4-oxo-2-(((R)-1-phenylethyl)amino)butanoic acid

Benzoylacrylic acid (35 g, 137 mmol) was dissolved in ethanol (400 mL) and (*R*) -1-phenylethylamine (18.3 g, 151 mmol) dissolved in ethanol was added (150 mL) The reaction mixture was stirred for 4 days at r.t. The solid was filtered off, washed with ethanol (125 mL), diethyl ether (50 mL) and air-dried. Amino acid was obtained as a colorless powder (47.6 g, 92 %, d.r. = 98: 2). mp = (177.4-178.6) ° C, $[\alpha]^{D}_{25}$ = -37.9 (c 1.0, MeOH / HCl)
^{1}H-NMR (300 MHz, acetone) δ 9.34 (s, 5H), 7.97 – 7.84 (m, 2H), 7.77 – 7.68 (m, 1H), 7.62 (dd, J = 7.5, 1.6 Hz, 2H), 7.50 – 7.31 (m, 4H), 4.82 (q, J = 6.8 Hz, 1H), 4.12 (t, J = 4.6 Hz, 1H), 3.95 – 3.77 (m, 2H), 3.30 (s, 1H), 1.78 (d, J = 6.9 Hz, 3H). ^{13}C-NMR (75 MHz, acetone) δ 206.88, 195.12, 169.37, 138.51, 137.10, 136.63, 131.64, 131.57, 130.26, 130.06, 129.41, 128.22, 123.14, 59.80, 39.83, 20.88.

Preparation of (*R*)-4-(3-bromophenyl)-2-(2-chloro-N-((*R*)-1-phenylethyl)acetamido)-4-oxobutanic acid

Amino acid (45g, 120 mmol) was suspended in DCM (500 mL). This mixture was cooled to 0 ° C in the ice bath, propylene oxide (115 g, 2 mol) was added and chloroacetyl chloride (15.5 g, 138 mmol) dissolved in DCM (75 mL) was added slowly over 1 h. The reaction mixture was further allowed to stir at RT for 1 h. DCM with propylene oxide was subsequently evaporated under reduced pressure. The resulting oil was dissolved in EtOAc (210 mL) and hexane (300 mL) was added to the mixture until the precipitation of product. This mixture was left in the freezer for 24 hours. The resulting crystals were filtered off, washed with cold hexane and air dried. N-chloroacetylated amino acid 54 was obtained as colorless crystals (36 g, 67%). mp = (144.0-146.1) ° C, $[\alpha]^{D}_{25}$ = +86.5 (c 1.0, MeOH)
^{1}H-NMR (300 MHz, cdcl3) δ 8.10 (s, 1H), 7.90 (d, J = 7.7 Hz, 1H), 7.69 (d, J = 7.8 Hz, 1H), 7.58 – 7.28 (m, 7H), 5.23 (q, J = 6.8 Hz, 1H), 4.62 (d, J = 5.5 Hz, 1H), 4.43 (dd, J = 18.2, 7.9 Hz, 1H), 4.22 (dd, J = 30.9, 12.4 Hz, 2H), 2.81 (dd, J = 18.3, 2.0 Hz, 1H), 2.04 (s, 1H), 1.64 (d, J = 6.9 Hz, 3H), 1.26 (t, J = 7.1 Hz, 1H). ^{13}C-

NMR (75 MHz, cdcl3) δ 195.69, 174.08, 166.94, 138.06, 137.58, 136.42, 131.43, 130.36, 128.80, 128.65, 128.08, 57.43, 52.08, 41.42, 41.09, 18.52.

Preparation of (2*R*,3*R*)-3-(3-bromobenzoyl)-5-oxo-1-((*R*)-1-phenylethyl)pyrrolidine-2-carboxylic acid

A solution of sodium hydroxide (9.3 g, 232 mmol) in water (600 mL) was prepared. To the solution was added N-chloroacetylated amino acid (35 g, 77.3 mmol) and dissolved. This reaction mixture was left to stand at RT for 24 h. Subsequently, the reaction mixture was acidified with 6 M HCl to pH 2. The resulting precipitate was filtered off, washed with water, cold methanol and dried. The filtered product was crystallized from methanol (400 mL). The resulting crystals were filtered off and washed with cold methanol. Cyclized amino acid was obtained as colorless crystals (23.3 g, 72%, d.r. = 99: 1). mp = (215.5-217.2) ° C, $[\alpha]^{D}_{25}$ = -29.1 (c 1.0, MeOH / NaOH)
^{1}H-NMR (300 MHz, dmso-d6) δ 8.27 – 8.14 (m, 1H), 8.08 – 8.00 (m, 1H), 7.91 (dd, J = 7.8, 2.0 Hz, 1H), 7.55 (t, J = 7.9 Hz, 1H), 7.45 – 7.17 (m, 5H), 5.03 (q, J = 7.1 Hz, 1H), 4.40 (d, J = 1.7 Hz, 1H), 4.33 (d, J = 9.4 Hz, 0H), 2.85 (dd, J = 16.7, 9.4 Hz, 1H), 2.53 (dd, J = 3.5, 1.8 Hz, 1H), 2.40 (dd, J = 16.8, 2.2 Hz, 1H), 1.54 (d, J = 7.0 Hz, 3H). ^{13}C-NMR (75 MHz, dmso-d6) δ 196.43, 172.22, 172.15, 139.74, 136.40, 136.29, 131.27, 131.13, 128.01, 127.71, 127.66, 127.32, 122.34, 59.47, 51.41, 42.82, 40.35, 40.08, 39.80, 39.52, 39.24, 38.96, 38.69, 33.23, 17.18.

Preparation of (2*R*,3*R*)-3-((*R*)-(3-bromophenyl)(hydroxy)methyl)-5-oxo-1-((*R*)-1-phenylethyl) pyrrolidine-2- carboxylic acid

Cyclized amino acid (10 g, 24 mmol) was suspended in methanol (200 mL). This slurry was cooled to 0 ° C in an ice bath. To this cooled mixture, sodium borohydride (2.73 g, 72 mmol) was added over 1h. Subsequently, cooling of the reaction mixture was stoped and the mixture was then allowed to stir until the temperature was equilibrated at room temperature. The mixture was evaporated under MeOH under reduced pressure and distilled water (100 mL) was added. This solution was acidified with 6 M HCl to pH 2. The resulting precipitate was filtered off, washed with water and dried. The filtered product was crystallized from methanol (800 mL). The resulting crystals were filtered off and washed with cold methanol. Cyclized hydroxy amino acid was obtained as colorless crystals (6 g, 60%, d.r. = 98: 2). mp = (288.7-289.7) ° C, $[\alpha]^{D}_{25}$ = -0.9 (c 1.0, MeOH / NaOH)
^{1}H-NMR (600 MHz, dmso-d6) δ 12.34 (s, 1H), 7.57 (t, J = 1.5 Hz, 1H), 7.46 (ddd, J = 7.9, 1.9, 1.0 Hz, 1H), 7.36 (d, J = 7.7 Hz, 1H), 7.33 – 7.20 (m, 4H), 5.05 (q, J = 7.1 Hz, 1H), 4.42 (d, J = 6.3 Hz, 1H), 4.13 (d, J = 1.1 Hz, 1H), 2.49 – 2.43 (m, 1H), 1.94 – 1.87 (m, 1H), 1.52 (d, J = 7.1 Hz, 2H). ^{13}C-NMR (151 MHz, dmso-d6) δ 173.21, 173.19 , 145.82, 139.87, 130.35, 130.15, 129.48, 127.95, 127.64, 127.23, 125.83, 121.59, 72.98, 59.19, 50.68, 43.36, 33.49, 16.97.

Preparation of (2*R*,3*R*)-methyl-3-((*S*)-(benzoyloxy)(3-bromophenyl)methyl)-5-oxo-1- ((*R*)-1-phenylethyl) pyrrolidine-2carboxylate

The esterified hydroxy acid (6 g, 13.9 mmol) was dissolved in dry THF (300 mL) and the resulting solution was cooled to -5 ° C. Benzoic acid (5.1 g, 41.6 mmol), PPh_3 (18.2 g, 69.4 mmol) was dissolved in this solution with stirring and the solution was placed under an argon atmosphere. After 30 minutes, diethyl azodicarboxylate (6g, 34.7 mmol) was added dropwise over half an hour, which was dissolved in 50 mL of THF and cooled in a freezer. The reaction mixture was allowed to stir at room temperature for 3 hours. Subsequently, tetrahydrofuran was evaporated in vacuo and the residue subjected to column chromatography. The hexane was used as the mobile phase and it was gradually transferred to the mobile phase, which was a 3: 2 mixture of hexane: ethyl acetate. The product obtained was re-subjected to column chromatography. The mixtures were diethyl ether: hexane (1: 2, 1: 1, 2: 1) and pure diethyl ether. Concentration of the appropriate fractions gave the esterified acid as a colorless powder (6.5 g, 87 %). mp = (132.4 - 133.7) ° C, $[\alpha]^{D}_{25}$ = +49.8 (c 1.0, MeOH)

^{1}H-NMR (300 MHz, CD_3OD) δ 8.11 – 8.03 (m, 2H), 7.71 – 7.15 (m, 13H), 6.10 (d, J = 5.3 Hz, 1H), 5.23 (q, J = 7.1 Hz, 1H), 4.18 (d, J = 1.9 Hz, 1H), 3.17 (s, 3H), 3.01 – 2.90 (m, 1H), 2.77 (dd, J = 17.2, 9.2 Hz, 1H), 2.53 (dd, J = 17.3, 2.4 Hz, 1H), 1.36 (d, J = 7.2 Hz, 3H). ^{13}C-NMR (75 MHz, CD_3OD) δ 176.25, 172.63, 166.57, 141.47, 139.90, 134.93, 132.78, 131.74, 130.78, 130.57, 129.89, 129.35, 129.24, 129.04, 126.37, 123.84, 77.67, 61.95, 52.71, 52.36, 42.75, 33.31, 16.66.

Preparation of (2*R*,3*R*)-methyl 3-((*R*)-(3-(hex-1-yne-1-yl)phenyl)(hydroxy)metyl)-5-oxo-1- ((*R*)-1-phenylethyl) pyrrolidine-2-carboxylate and (2*R*,3*R*)-methyl 3-((*S*)-(benzoyloxy)- (3-(hex-1-yne-1-yl)phenyl)methyl)-5-oxo-1-((*R*)-1-phenylethyl) pyrrolidine-2-carboxylate

The esterified acid (5.5 g, 10.2 mmol) was suspended in triethylamine (165 mL). $Pd(PPh_4)_3$ (1.18 g, 1.1 mmol) and CuI (0.194 g, 1.1 mmol) were then added to the suspension while stirring and the reaction mixture was placed under an argon atmosphere. After 45 minutes, hex-1-yne (2.53 g, 30.7 mmol) was added to the mixture and the mixture was heated to 65 ° C and stirred for 20 hours. Subsequently, TEA was evaporated under reduced pressure and the residue was subjected to column chromatography. 1: 9, 1: 1, 2: 1 and 3: 1 mixtures of diethyl ether: hexane as the eluent. The product was re-subjected to column chromatography. Ethyl acetate: hexanes (1: 9 and 3: 7) were used as mobile phases. The product was re-subjected to column chromatography. The mixture of diethyl ether: hexane (1: 9, 1: 4 and 1: 1) was used as the eluent. Concentration of the appropriate fractions gave product as a colorless powder (4.9 g, 89 %). mp = (59.0 - 64.1) ° C, $[\alpha]^{D}_{25}$ = +45.0 (c 1.0, MeOH)
1H-NMR (300 MHz, CD_3OD) δ 8.11 (dd, J = 8.4, 1.4 Hz, 1H), 7.75 – 7.65 (m, 1H), 7.57 (ddd, J = 8.2, 6.7, 1.3 Hz, 1H), 7.47 – 7.20 (m, 5H), 6.12 (d, J = 5.4 Hz, 1H), 5.27 (q, J = 7.1 Hz, 1H), 4.22 (d, J = 2.0 Hz, 1H), 3.22 (s, 2H), 3.05 – 2.92 (m, 1H), 2.81 (dd, J = 17.2, 9.2 Hz, 1H), 2.57 (dd, J = 17.3, 2.4 Hz, 1H), 2.45 (t, J = 6.8 Hz, 1H), 1.69 – 1.45 (m, 3H), 1.40 (d, J = 7.2 Hz, 2H), 0.99 (t, J = 7.2 Hz, 2H). 13C-NMR (151 MHz, CD_3OD) δ 176.31, 172.71, 166.63, 139.91, 139.17, 134.85, 132.60, 130.77, 130.72, 130.43, 129.95, 129.87, 129.34, 129.27, 129.03, 126.67, 126.19, 91.99, 81.04, 78.07, 61.96, 52.68, 52.36, 42.80, 33.40, 31.92, 23.01, 19.66, 16.70, 13.95.

Conclusions

Constrained analogs of HPA-12 were successfully prepared in high diastereomeric purity and in good yields. Final constrained analogs of HPA-12 were sent for testing their affinity to the CERT protein. Preliminary results France show EC_{50} for CERT comparable with those for HPA-12.

Acknowledgments

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Conflicts of Interest:

The authors declare no conflict of interest.

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