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Stereoselective ring contraction of 2,5-diketopiperazines: An innovative approach to the synthesis of promising bioactive 5-membered scaffolds

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ABSTRACT

Ring contraction of 2,5-diketopiperazines by TRAL-alkylation led us to the stereoselective synthesis of original pyrrolidine-2,4-diones, a novel series of promising molecules with moderate anti-proliferative activity on breast cancer cells.

1. Introduction

The discovery of new molecular scaffolds, especially small ring compounds or heterocyclic moieties, is crucial in drug discovery. In regular medicinal chemistry practices, or in lead optimization processes, ring-chain transformations are universal analogical approaches to reach biologically more potent ligands, structurally close to the original active structure. For example, a direct access to designed ring-contracted scaffolds could be critical to convert a hit into a new library of derivatives when investigating higher selectivity within a given target family [1,2].

We recently described a helpful stereoselective reaction, the TRAL-alkylation, allowing the ring contraction of Boc-activated 2,5-diketopiperazines (DKPs) into pyrrolidine-2,4-diones (Fig. 1) [3–5]. This tandem rearrangement-alkylation strategy is an excellent stereoselective ring-to-ring method, in line with the concept of homology supported by medicinal chemistry [2]. This deliberative conversion of the more readily accessible heterocyclic system leads to pertinent 5-membered heterocycles, with bio-chemical improvement of the architecture of the final compound. The skeleton of the final synthon is condensed with a concomitant extrusion of one heteroatom.

We report here an efficient access to a series of original 3-substituted 2,5-diketopiperazines, acting as building blocks for the ring transformation into pyrrolidine-2,4-diones. Interestingly, this novel series of compounds exhibited potentially anti-proliferative activity on human breast cancer cell line SKBR3. Moreover, their utility as starting material for transannular rearrangement into biologically more potent 5-membered heterocycles was demonstrated.

2. Results and discussions

2.1. Synthesis and biological evaluation of original 3-substituted 2,5-diketopiperazines as potential anti-proliferative agents of cancer cells

The smallest cyclic peptides, commonly called diketopiperazines, are an attractive class of scaffolds in drug research, due to its chiral nature, rigid structure, resistance to proteolysis and presence of donor/acceptor groups for hydrogen bonds formation [6]. The DKP moiety, present in several natural and synthetic products, is biologically active on various pharmacological targets, displaying antihyperglycaemic, antiviral, antitumour, antifungal and antibacterial activities [6–15]. Instigator of the transannular rearrangement of activated lactam (TRAL) [3], we became deeply interested by a facile and efficient route to 3-substituted 2,5-diketopiperazines *cyclo*-[Gly-Xaa] (Fig. 2). Moreover, some DKPs and DKP derivatives having proved their ability to inhibit cancer cell growth [8,9,12,14,16], we consequently decided to investigate their potential activity as anti-proliferative agents against human SKBR3 breast cancer cell line.

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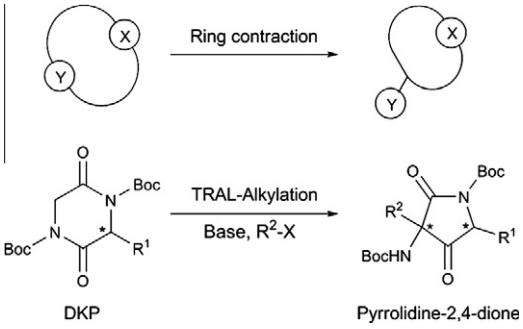


Fig. 1. General theory of the ring contraction matching with the representation of the TRAL-alkylation.

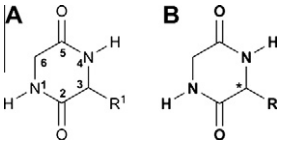
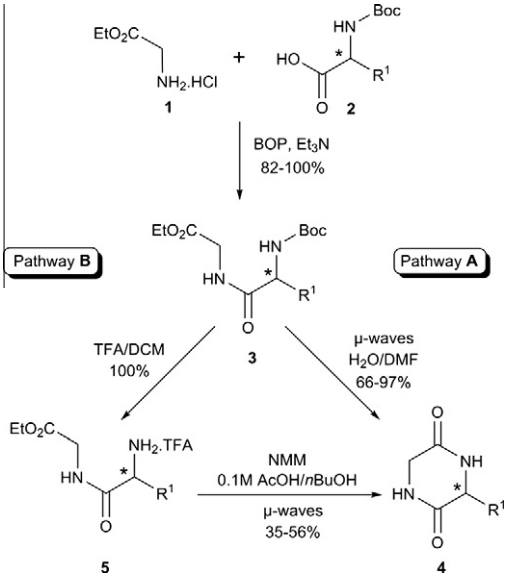


Fig. 2. General formula of 3-disubstituted 2,5-diketopiperazine (A) and Cyclo-[Gly-Xaa] (B).

Having identified and evaluated all the described synthetic methods for the synthesis of 2,5-diketopiperazine derivatives [17–21], it appeared that for an efficient access to enantiopure *cyclo*-[Gly-Xaa], ethyl ester of glycine was the best ester derivative required for a high stability and reproducibility of the cyclization step (Scheme 1). The di-protected acyclic peptide Boc-Xaa-Gly-OEt **3** was obtained in excellent yields by a conventional peptide coupling between the hydrochloride salt of glycine ethyl ester **1** and the corresponding Boc-protected amino acid **2**, using BOP reagent and triethylamine (Scheme 1) [22,23]. Applying the optimal cyclization protocol recently developed in our laboratory for the synthesis of the unique *cyclo*-[(^{15}N)Gly-Val] [24], and opting for microwave-assisted heating in a mixture of water and DMF as solvents, we were able to synthesize a small library of 3-substituted 2,5-diketopiperazines (Scheme 1, pathway A). Microwaves conditions allowed here a reproducible one-pot Boc deprotection and



Scheme 1. General synthesis of 3-substituted 2,5-diketopiperazines.

cyclization of the linear dipeptide **3** to access to synthetically familiar DKPs **4a**–**e** or innovative DKPs **4f**–**k**, with good to excellent yields (Table 1, entries 1–11) [20].

According to HPLC analysis on chiral phase, cyclic compounds were obtained with total retention of the initial stereochemical configuration of **2**. In each reaction, we could note the presence of a variable amount of the unprotected dipeptide as by-product *H-Xaa-Gly-OH*, due to the assisted microwave heating. Unpredictably, when the side chain of the aminoacid Xaa initially present on the starting material **3** contains a protected amine or an ester function, no trace of the cyclic peptide could be detected. The starting material **3** was then partially or totally deprotected leading generally to *H-Xaa-Gly-OEt* (80%) and *H-Xaa-Gly-OH* (20%).

We then decided to go back to a more conventional synthetic strategy, the two-step route described by Johnson et al. [25]. Refluxing conditions were improved by microwave irradiation in order to reduce time, by-products, and to increase efficiency of the process. First of all, the Boc protection was removed at room temperature to afford in quantitative yield the TFA salt of the *H-Xaa-Gly-OEt* (Scheme 1, pathway B). The cyclization reaction conditions on microwaves were optimized using various experimental conditions such as temperature, reaction time, power of the irradiation and concentration of starting materials. A control reaction was also performed under normal thermal heating in refluxing to achieve the same completion. Under optimal conditions, DKPs **4j**–**k** were obtained in good yield, in 1 h instead of 6 h under conventional conditions (Scheme 1, pathway B), after purification by a simple washing step of the crude product.

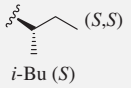
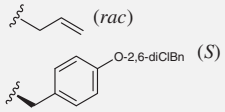
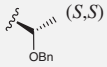
The nature of the side chain of the Xaa residue had a huge influence on the reactivity of the linear dipeptide. Pathways A or B were chosen for the synthesis of *cyclo*-[Gly-Xaa], depending on the presence or the absence of reactive side chain functions. When a reactive function such as an ester or an amine was present, pathway B was followed, if unreactive functions such as an alkyl chain or an ether oxide were present, the fastest pathway A was preferred (Scheme 1). Regarding the anticancer activity described by C. Frost et al. on DKPs including **4b**–**d** [12,16], our small library of **4a**–**l** (Table 1) was then evaluated for its potential inhibition of proliferation on SKBR3 breast cancer cell line using a CellTiter-Glo™ Luminescent Cell Viability Assay. Inhibitions that were observed were disappointing, lower than expected (Table 1). At 100 μM the greatest anti-proliferative activity being noted was for *cyclo*-[Gly-Ser(OBn)] at 36% inhibition.

2.2. Ring contraction of valuable 3-substituted 2,5-diketopiperazines into original 5-membered scaffolds

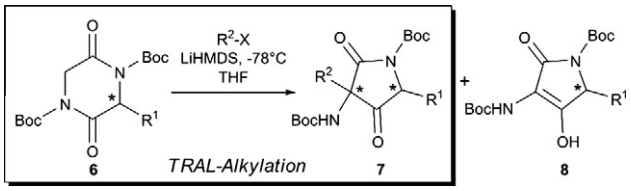
Keeping in mind the idea to transform a 6-membered scaffold into its contracted moiety, we have imagined an access to heterocyclic platforms with biological potentials. We hoped here to improve the low anti-proliferative activities founded with DKP moieties by designing close analogues with global structural similarities. Starting from suitable Boc-activated DKP **6**, we aimed to exploit the alkylated version of the transannular rearrangement of activated lactams, in order to reach, in a stereocontrolled manner, functionalized pyrrolidine-2,4-diones **7** (Scheme 2).

To the best of our knowledge, the TRAL-alkylation tandem, starting from unsymmetrical DKPs, was just implemented on appropriate *bis*-Boc derivatives of *cyclo*-[Gly-Val] and *cyclo*-[Gly-Leu], with only few alkylating agents. None of the side chains of the described DKPs were suitable for further functionalizations, and only one of the alkylating agents was functionalized, limiting future derivatization of the final heterocyclic scaffold. Besides the proof of concept of homology, we wish then to provide here more information about the reactivity of activated DKPs and the feasibility of the TRAL-alkylation.

Table 1
DKPs synthesis and biological evaluation.

Entry	4	R^1[a]	Xaa	% Literature yields[b]	% Yields[c]	% Inhibition observed[d]
1	**4a**	Me(*S*)	Ala	98	97 (A)	30
2	**4b**	*i*-Pr(*R*)	*D*-Val	87	93 (A)	Inactive (22)[e]
3	**4c**	 (*S*,*S*) *i*-Bu (*S*)	Ile	91 (*dr* 9:1)	66 (A)	Inactive (24)[e]
4	**4d**	*i*-Bu(*S*)	Leu	89	90 (A)	5 (27)[e]
5	**4e**	Bn(*S*)	Phe	82	94 (A)	22
6	**4f**	Bn (*S*) (*rac*)	*D*,*L*-AllylGly	–	84 (A)	Inactive
7	**4g**	 O-2,6-diClBn (*S*)	Tyr(O-2,6-diClBn)	–	86 (A)	8
8	**4h**	$CH_2OBn(S)$	Ser(OBn)	–	74 (A)	36
9	**4i**	 (*S*,*S*)	Thr(OBn)	–	73 (A)	Inactive
10	**4j**	$CH_2CO_2Bn(S)$	Asp(OBn)	–	56 (B)	Inactive
11	**4k**	$(CH_2)_4NHZ(S)$	Lys(Z)	–	35 (B)	32

[a] Configuration of the stereogenic centers are specified into parentheses.
[b] Yields described in the literature using a domestic microwave oven [20].
[c] Overall yields of DKPs isolated after flash chromatography (pathway A or B).
[d] Growth inhibition values on SKBR3 cancer cell line at 100 μM concentration.
[e] Values into parentheses represent the mean percentage inhibition on the growth of MCF-7 cells at a concentration of 100 μM described by Frost et al. [12,16].



Scheme 2. General synthesis of functionalized pyrrolidine-2,4-diones.

We first decided to use the bis-Boc-*cyclo*-[Gly-Val], as a model substrate for our methodological study, investigating new alkylating entities (Table 2, entries 1–11). Unfortunately, the heterogeneous yields observed did not allow to suggest a rational and predictable chemical reactivity for the TRAL-alkylation. In some cases (entries 3, 4, 7–10), only the rearrangement occurred, leading to the non-alkylated aminotetramates **8** in low yield, the starting material **6** being always the major compound. For example, it was unexpected to carry out successfully the tandem reaction on *cyclo*-[Gly-Val] **6b** with the allylbromide and to synthesize only the aminotetramate **8b**, when using propargylbromide. Various other working conditions such as stoichiometric adaptations, temperature variations, use of various bases (*t*BuOK, NaH), were investigated, with no improvement. We postulated here that the presence of an acidic proton on the alkylating entity or on the side chain of the DKP could significantly influence the rates of the product of TRAL-alkylation. Nevertheless, examination of the reaction with various substituted DKPs or different alkylated reagents allowed us to synthesize innovative compounds (entries 5, 6, 13, 17) with acceptable yields, regarding the remarkable stereoselectivity of the reaction and the originality of the heterocycle moiety fashioned.

When required, chemoselective deprotection of the *N*-Boc group could subsequently be easily achieved on the series of pyrrolidine-2,4-diones. The *N*-Boc was preferentially and firstly removed from the lactam function at 0 °C using mild acidic conditions, while the second *N*-Boc group was cleaved at room temperature with higher amount of TFA to afford the free amine **10** in a quantitative yield (Scheme 3).

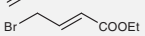
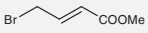
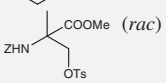
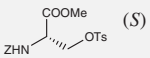
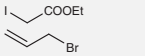
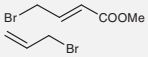
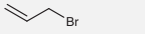
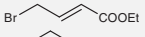
Our methodological studies led us to gain access to some promising functionalized pyrrolidine-2,4-diones. As an illustration, the synthesis of the synthon **7c** could be extended to the construction of innovative spiroheterocycles. This type of framework is of great interest for medicinal chemists, due to its sterically constrained feature and to its implication on biological systems [26–36]. Toward the aim to develop original bio-chemical skeletons, we took a real curiosity in the stereocontroled synthesis of such derivatives. After a rapid and quantitative deprotection of the *bis*-Boc protecting groups of **7c** to yield **10c**, we were able to access to the 2,6-diazaspiro[4,5]decane-1,4,7-trione **11** in a one-pot strategy after reduction of the exocyclic double bond followed by an intramolecular cyclization, in 81% yield (Scheme 4). The creation of **11**, an original aza-spirocycle, is of great interest regarding the similarity of such structurally intricate structure in alkaloids or in a variety of biological active compounds [26–40].

At this point, we decided to explore the anti-proliferative activities of our new compounds on breast cancer cell line SKBR3 to make a comparison with the data previously obtained with the DKPs (Fig. 3). We were pleased to observe that none of the 5-membered rings were less active than their originally expanded related compounds. Moreover, when DKP **4b** completely failed to inhibit growth of cancer cells, contracted homologues **7**, **9** or **10** exhibited a noticeable inhibitory effect. While the more constrained ring **11** was totally inactive, we could assume that the extrusion of the amine function from the ring, instead of its inclusion into a lactam, potentiated by the presence of an alkylated chain, are critical for the biological activity. Further studies are currently devoted to the confirmation of this postulate in concomitance with the optimization of this first hit.

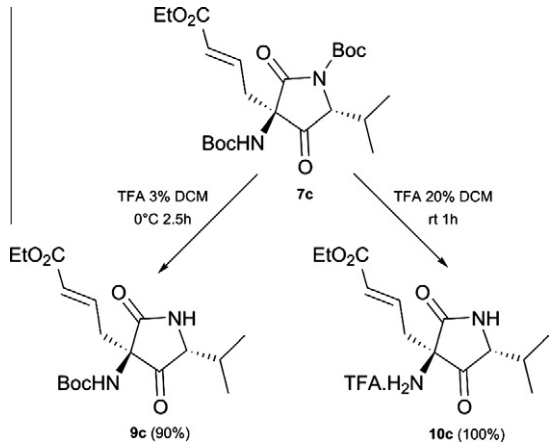
3. Conclusion

We described here an efficient, rapid and enantioconservative access to 3-substituted 2,5-diketopiperazines with good to excellent yields, depending on the nature of the side chain of the amino acid involved. Looking for more potent compounds, we decided to apply the alkylating version of our innovative stereoselective ring contraction reaction to a small library of original DKPs, using

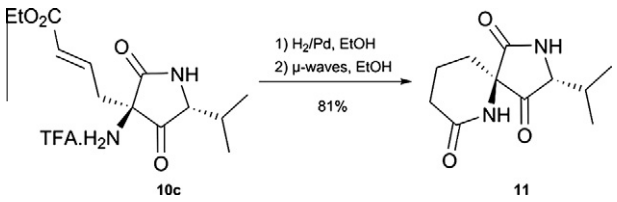
Table 2
TRAL/alkylation on activated DKPs.

Entry	6	Xaa	R^2-X[a]	7	% Yields[b]	8	% Yields[b]
1	**6b**	*D*-Val		**7a** [3]	76 [3]	**8b** [3]	20
2	**6b**	*D*-Val		**7a**	82	**8b** [3]	15
3	**6b**	*D*-Val		**7b**	0	**8b** [3]	20
4	**6b**	*D*-Val		**7b**	0	**8b** [3]	20
5	**6b**	*D*-Val		**7c**	42[a]	**8b** [3]	25
6	**6b**	*D*-Val		**7d**	49[a]	**8b** [3]	20
7	**6b**	*D*-Val		**7e**	<5[d]	**8b** [3]	15
8	**6b**	*D*-Val		**7f**	0	**8b** [3]	15
9	**6b**	*D*-Val		**7g**	**0**[c]	**8b** [3]	40
10	**6b**	*D*-Val		**7g**	0[c]	**8b** [3]	40
11	**6b**	*D*-Val		**7h** [3]	70 [3]	**8b** [3]	25
12	**6f**	*D,L*-AllylGly		**7i**	<5[d]	**8f**	<5[d]
13	**6f**	*D,L*-AllylGly		**7j**	35	**8f**	<5[d]
14	**6h**	Ser(OBn)		**7k**	<5[d]	**8h**	<5[d]
15	**6h**	Ser(OBn)		**7l**	<5[d]	**8h**	<5[d]
16	**6h**	Ser(OBn)		**7m**	<5[d]	**8h**	<5[d]
17	**6i**	Thr(OBn)		**7n**	30	**8i**	<5[d]
18	**6j**	Asp(OBn)		**7o**	<5[d]	**8j**	<5[d]
19	**6j**	Asp(OBn)		**7p**	<5[d]	**8j**	<5[d]

[a] Ethyl-4-bromocrotonate could be purchased with a maximal purity of 80% while methylester derivatives could be purchased with a maximal purity of 90%.
[b] Yields of isolated compounds after flash chromatography.
[c] Using the experimental basic conditions for the TRAL-alkylation, only the product of β-elimination of the starting material R^2X was formed.
[d] Yield based on LC–MS analysis, product not isolated.



Scheme 3. Chemoselective and total *N*-Boc deprotection of functionalized pyrrolidine-2,4-dione **7c**.



Scheme 4. Access to an innovative diaza-spiro [4,5].

functionalized alkylating reagents to enlarge molecular diversity. Although the TRAL-alkylation strategy was found so far not to be applicable to a large variety of starting materials, we managed to successfully access to pyrrolidine-2,4-diones moderately inhibiting growth of cancer cells, and to achieve the synthesis of an original spiroheterocycle of potential interest in biological applications.

4. Material and methods

4.1. General methods

All solvents were dried and freshly distilled before use. Reactions were magnetically stirred and monitored by thin layer chromatography using Merck-Kieselgel 60 F254 plates. Visualisation was accomplished with UV light and exposure to a 10% solution of ninhydrin in ethanol followed by heating. Chromatography columns were performed using Merck-Kieselgel 60 (230–400 mesh). Melting points were recorded on a Buchi 510. HPLC analyses were performed on a Waters-Enpower Pro (column 50×4.6 mm Chromolith SpeedRod RP-18, detection UV). Mass spectra were obtained on a Micromass Q-Tof mass spectrometer using electrospray ionization. ^{1}H NMR spectra were recorded at ambient temperature on Bruker 200 MHz, Bruker Advance 300 MHz or Bruker A DRX 400 MHz spectrometers. Chemicals shifts (δ) are reported from tetramethylsilane with the solvent resonance as the internal standard. Data are reported as follows: chemical shift (δ), multiplicity (s = singulet, d = doublet, t = triplet, br = broad, m = multiplet), coupling constants (*J*/Hz), integration, and assignment. ^{13}C NMR spectra were recorded at ambient temperature on Bruker Advance 300 MHz or Bruker A DRX 400 MHz spectrometers. Chemicals shifts (δ) are reported from tetramethylsilane with the solvent resonance as the internal standard. Data are reported as follows:

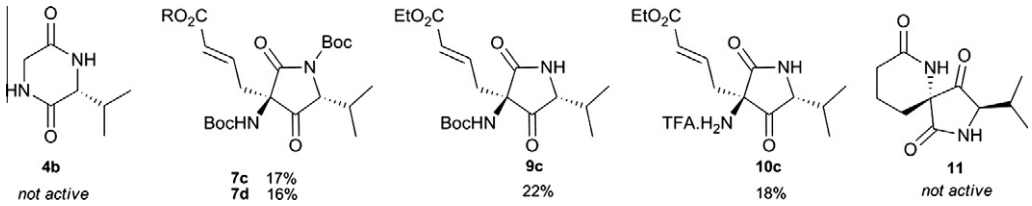


Fig. 3. Percent of inhibition on the growth of SKBR3 cell line at a 25 μM concentration of compounds.

chemical shift (δ) and assignment. The reported ^{1}H NMR signals were assigned using standard 2D NMR techniques or by direct comparison to the ^{1}H NMR spectra of corresponding starting materials. The reported ^{13}C NMR signals were assigned using DEPT-135 and HMQC techniques or by direct comparison to the ^{13}C NMR spectra of corresponding starting materials.

4.2. General experimental procedure for the synthesis of Boc-Xaa-Gly-OEt dipeptides 3

To a solution of Boc-Xaa-OH **2** (1 eq.) in anhydrous DMF were sequentially added H-Gly-OEt.HCl **1** (1 eq.), BOP reagent (1 eq.), and then triethylamine dropwise (3 eq.). The reaction mixture was stirred under argon atmosphere at room temperature for 12 h. EtOAc was then added to the reaction media. The organic layer was sequentially washed with 0.1 N HCl solution, saturated aqueous $NaHCO_3$ solution, dried over $MgSO_4$ and evaporated to dryness to afford the desired dipeptide **3** in 82–100% yield.

4.3. General experimental procedure for the synthesis of Boc-deprotected dipeptide 5

Deprotection of Boc moeties were carried out with TFA 50% in anhydrous CH_2Cl_2 at room temperature for 1 h. Then, the reaction media was evaporated to dryness, and the remaining TFA was coevaporated with toluene to afford the desired TFA salt **5** in quantitative yield.

4.4. General experimental procedures for the synthesis of Cyclo-[Gly-Xaa]

4.4.1. Pathway A

Boc-Xaa-Gly-OEt dipeptide **3** was dissolved in DMF (1 Vol.). Water (~10 Vol.) was then added to this solution (a precipitation of the substrate might occur). The reaction media was heated in a microwave oven at 150 °C for 2 h 30 min. The reaction mixture was then evaporated to dryness under vacuum. The crude material was filtered on silica gel (elution by CH_2Cl_2:MeOH 95:5), and the filtrate was evaporated under vacuum to afford the desired diketopiperazine **4** in 66–97% yield.

4.4.2. Pathway B

The TFA salt of H-Xaa-Gly-OEt **5** was dissolved in butan-1-ol containing 0.1 M of acetic acid. After addition of *N*-methylmorpholine (1 eq.), the reaction mixture was irradiated in a microwave oven at 150 °C for 1 h. The reaction media was then concentrated, and a minimum amount of CH_2Cl_2 was added. A rapid filtration afforded the pure diketopiperazine **4** in 35–56% yield.

4.4.3. Cyclo-[Gly-Ala] **4a**

Following the general procedure, pathway A, compound **4a** was isolated as white crystals (97%), mp 226 °C. δ_H (300 MHz; CD_3OD; Me_4Si) 1.34 (d, 3H, J = 7.0 Hz, H_{CH*CH3}), 3.82–3.88 (m, 2H, H_{NHCH2}), 3.90–3.95 (m, 1H, H_{CH*}); δ_C (75 MHz, CD_3OD; Me_4Si) δ 17.8 (C_{CH*CH3}), 44.5 (C_{NHCH2}), 50.7 (C_{CH*}), 166.5 (C_{CO}), 169.5 (C_{CO}); *m/z* (ESI$^+$) 129.1 (HRMS-ESI$^+$) 129.0676 ($[M + H]^+$ $C_5H_9N_2O_2$ requires 129.0664).

4.4.4. Cyclo-[Gly-Val] **4b**

Following the general procedure, pathway A, compound **4b** was isolated as white crystals (93%), mp 260 °C. δ_H (300 MHz, DMSO-d6; Me_4Si) δ 0.86 (d, 3H, J = 6.8 Hz, H_{CH3CH}), 0.93 (d, 3H, J = 7.0 Hz, H_{CH3CH}), 2.06–2.15 (m, 1H, H_{CH3CH}), 3.53 (m, 1H, H_{CH*}), 3.62 (d, 1H, J = 17.7 Hz, H_{CH2}), 3.82 (d, 1H, J = 17.7 Hz, H_{CH2}), 8.02 (br s, 1H, H_{NH}), 8.20 (br s, 1H, H_{NH}); δ_C (75 MHz, DMSO-d6; Me_4Si) δ 17.0 ($C_{(CH3)2CH}$), 18.5 ($C_{(CH3)2CH}$), 32.2 ($C_{(CH3)2CH}$), 44.1 (C_{CH2}), 59.7 (C_{CH*}), 166.0 (C_{CO}), 167.2 (C_{CO}) (data consistent with commercial product).

4.4.5. Cyclo-[Gly-Ile] **4c**

Following the general procedure, pathway A, compound **4c** was isolated as white crystals (66%), mp 240 °C. δ_H (300 MHz, CD_3OD; Me_4Si) δ 0.86 (t, 3H, J = 7.4 Hz, H_{CH2CH3}), 0.92 (d, 3H, J = 7.0 Hz, H_{CH*CH3}), 1.09–1.24 (m, 1H, H_{CH*CH2}), 1.39–1.51 (m, 1H, H_{CH*CH2}), 1.81–1.90 (m, 1H, H_{CH*CH3}), 3.70–3.76 (m, 1H + 1H, H_{NHCH2} + H_{NHCH*}), 3.89 (dd, 1H, J = 0.8 Hz, J = 18.2 Hz, H_{NHCH2}); δ_C (75 MHz, CD_3OD; Me_4Si) δ 11.5 (C_{CH2CH3}), 14.9 (C_{CH*CH3}), 25.0 (C_{CH*CH2}), 40.7 (C_{CH*CH3}), 44.8 (C_{NHCH2}), 60.5 (C_{NHCH*}), 168.2 (C_{CH2CO}), 169.6 (C_{CH*CO}); *m/z* (ESI$^+$) 171.1 (HRMS-ESI$^+$) 171.1142 ($[M + H]^+$ $C_8H_{15}N_2O_2$ calculated 171.1134).

4.4.6. Cyclo-[Gly-Leu] **4d**

Following the general procedure, pathway A, compound **4d** was isolated as white crystals (90%), mp 217 °C. δ_H (300 MHz, CD_3OD; Me_4Si) δ 0.86 (d, 3H, J = 5.8 Hz, H_{CHCH3}), 0.88 (d, 3H, J = 5.9 Hz, H_{CHCH3}), 1.55–1.60 (m, 2H, H_{CH*CH2}), 1.66–1.77 (m, 1H, $H_{CH(CH3)2}$), 3.73 (dd, 1H, J = 0.7 Hz, J = 17.8 Hz, H_{NHCH2}), 3.80 (t, 1H, J = 6.9 Hz, H_{NHCH*}), 3.91 (dd, 1H, J = 0.8 Hz, J = 17.8 Hz, H_{NHCH2}); δ_C (75 MHz, CD_3OD; Me_4Si) δ 21.7 ($C_{CH(CH3)2}$), 23.0 (C_{CHCH3}), 24.9 (C_{CHCH3}), 43.5 (C_{CH*CH2}), 44.9 (C_{NHCH2}), 54.4 (C_{CH*}), 168.5 (C_{CH*CO}), 171.2 (C_{CH2CO}); *m/z* (ESI$^+$) 171.1 (HRMS-ESI$^+$) 171.1143 ($[M + H]^+$ $C_8H_{15}N_2O_2$ requires 171.1134).

4.4.7. Cyclo-[Gly-Phe] **4e**

Following the general procedure, pathway A, compound **4e** was isolated as white crystals (94%), mp 240 °C, t_r = 0.899 min. δ_H (300 MHz, DMSO-d6; Me_4Si) δ 2.76 (d, 1H, J = 17.4 Hz, H_{NHCH2}), 2.89 (dd, 1H, J = 4.9 Hz, J = 13.5 Hz, H_{CH*CH2}), 3.10 (dd, 1H, J = 4.4 Hz, J = 13.4 Hz, H_{CH*CH2}), 3.36 (m, 1H, H_{CH*CH2}), 4.06 (m, 1H, H_{CH*}), 7.17–7.29 (m, 5H, H_{arom}), 7.90 (s, 1H, H_{NH}), 8.16 (s, 1H, H_{NH}); δ_C (75 MHz, DMSO-d6; Me_4Si) δ 38.7 (C_{CH*CH2}), 43.6 (C_{NHCH2}), 55.4 (C_{CH*}), 126.7–135.9 (C_{arom}), 165.6 (C_{CH*CO}), 167.1 (C_{CH2CO}); *m/z* (ESI$^+$) 205.1 (HRMS-ESI$^+$) 205.0985 ($[M + H]^+$ $C_{11}H_{13}N_2O_2$ calculated 205.0977).

4.4.8. Cyclo-[Gly-AllylGly] **4f**

Following the general procedure, pathway A, compound **4f** was isolated as white crystals (84%), mp 199 °C. δ_H (300 MHz, CD_3OD; Me_4Si) δ 2.48 (m, 2H, H_{CH2}—$_{CH}$=$_{CH2}$), 3.74 (dd, 1H, J = 17.9 Hz, J = 1.1 Hz, H_{CH2}—$_{NH}$), 3.84 (dd, 1H, J = 17.9 Hz, J = 1.1 Hz, H_{CH2}—$_{NH}$),

3.92 (t, 1H, J = 4.7 Hz, H_{CH*CH2}), 5.08–5.14 (m, 2H, $H_{CH=CH2}$), 5.65–5.76 (m, 1H, $H_{CH=CH2}$); δ_C (75 MHz, CD_3OD; Me_4Si) δ 37.1 ($C_{CH2-CH*}$), 44.2 (C_{CH2Gly}), 53.9 (C_{CH*}), 119.0 ($C_{CH2=CH}$), 132.9 ($C_{CH=CH2}$), 165.9 ($C_{COlactam}$), 167.2 ($C_{COlactam}$); m/z (ESI^-) 153.1 (HRMS-ESI^-) 153.0659 ($[M-H]^-$ $C_7H_9N_2O_2$ calculated 153.0664).

4.4.9. Cyclo-[Gly-Tyr(O-2,6-diCl-Bn)] **4g**

Following the general procedure, pathway A, compound **4g** was isolated as white crystals (86%), mp 202 °C, t_r = 1.902 min. δ_H (300 MHz, DMSO-d6; Me_4Si) δ 2.51–2.54 (m, 1H, H_{CH*CH2}), 2.81–2.89 (m, 1H, H_{NHCH2}), 3.06 (dd, 1H, J = 4.4 Hz, J = 13.5 Hz, H_{NHCH2}), 3.36–3.43 (m, 1H, H_{CH*CH2}), 4.03 (m, 1H, H_{CH*}), 5.20 (s, 2H, H_{OCH2}), 6.98–7.58 (m, 7H, H_{arom}), 7.90 (s, 1H, H_{CH*NH}), 8.15 (m, 1H, H_{CH2NH}); δ_C (75 MHz, DMSO-d6; Me_4Si) δ 37.9 (C_{CH*CH2}), 43.7 (C_{NHCH2}), 55.6 (C_{OCH2}), 64.9 (C_{CH*}), 114.3–157.4 (C_{arom}), 165.6 (C_{CH2CO}), 167.2 (C_{CH*CO}); m/z (ESI^+) 379.1 (HRMS-ESI^+) 379.0617 ($[M+H]^+$ $C_{18}H_{17}N_2O_3Cl_2$ calculated 379.0616).

4.4.10. Cyclo-[Gly-Ser(OBn)] **4h**

Following the general procedure, pathway A, compound **4h** was isolated as a yellowish powder (74%), mp 157 °C, t_r = 1.112 min. δ_H (300 MHz, CD_3OD; Me_4Si) δ 3.67 (dd, 1H, J = 9.7 Hz, J = 2.7 Hz, H_{CH2CH*}), 3.78 (d, 1H, J = 17.4 Hz, H_{CH2NH}), 3.93 (dd, 1H, J = 9.7 Hz, J = 2.7 Hz, H_{CH2CH*}), 3.98 (d, 1H, J = 17.4 Hz, H_{CH2NH}), 4.02 (m, 1H, H_{CH*CH2}), 4.56 (s, 2H, $H_{CH2Phenyl}$), 7.26–7.38 (m, 5H, H_{arom}); δ_C (75 MHz, CD_3OD; Me_4Si) δ 45.1 (C_{CH2NH}), 56.9 (C_{CH*CH2}), 72.3 (C_{CH2CH*}), 74.0 ($C_{CH2Phenyl}$), 127.5–138.7 (C_{arom}), 169.1 ($C_{COlactams}$); m/z (ESI^+) 235.1 (HRMS-ESI^+) 235.1077 ($[M+H]^+$ $C_{12}H_{15}N_2O_3$ calculated 235.1083).

4.4.11. Cyclo-[Gly-Thr(OBn)] **4i**

Following the general procedure, pathway A, compound **4i** was isolated as white crystals (73%), mp 180 °C, t_r = 1.186 min. δ_H (300 MHz, CD_3OD; Me_4Si) δ 1.28 (d, 3H, J = 6.4 Hz, H_{CHCH3}), 3.63 (d, 1H, J = 17.3 Hz, $H_{NHCH2CO}$), 3.76 (s, 1H, H_{CH*}), 3.88 (d, 1H, J = 17.3 Hz, $H_{NHCH2CO}$), 4.11 (m; 1H, H_{CHCH3}), 4.42 (d, 1H, J = 11.3 Hz, H_{CH2Ph}), 4.64 (d, 1H, J = 11.3 Hz, H_{CH2Ph}), 7.31 (m, 5H, H_{C6H5}); δ_C (75 MHz, CD_3OD; Me_4Si) δ 14.6 (C_{CH3}), 44.1 (C_{NHCH2}), 60.3 (C_{CH*}), 70.9 (C_{CHCH3}), 76.3 ($C_{CH2C6H5}$), 127.3–138.13 (C_{arom}), 168.6 (C_{CO}), 169.2 (C_{CO}); m/z (ESI^+) 249.1 (HRMS-ESI^+) 249.1234 ($[M+H]^+$ $C_{13}H_{17}N_2O_3$ calculated 249.1239).

4.4.12. Cyclo-[Gly-Asp(OBn)] **4j**

Following the general procedure, pathway B, compound **4j** was isolated as a white crystal (56%), mp 164 °C, t_r = 1.189 min. δ_H (300 MHz, DMSO-d6; Me_4Si) δ 2.76 (dd, 1H, J = 16.9 Hz, J = 5.3 Hz, $H_{CH2CO2Bn}$), 2.87 (dd, 1H, J = 16.9 Hz, J = 5.0 Hz, $H_{CH2CO2Bn}$), 3.67 (d, 1H, J = 17.4 Hz, H_{CH2NH}), 3.79 (d, 1H, J = 17.2 Hz, H_{CH2NH}), 4.19 (m, 1H, H_{CHCH2}), 5.11 (s, 2H, H_{CH2Bn}), 7.37 (m, 5H, H_{C6H5}), 8.09 (s, 1H, H_{NH}), 8.17 (s, 1H, H_{NH}); δ_C (75 MHz, DMSO-d6; Me_4Si) δ 36.4 ($C_{CH2CO2Bn}$), 44.5 (C_{CH2NH}), 50.7 (C_{CHCH2}), 65.7 (C_{CH2Bn}), 127.9–135.9 (C_{C6H5}), 165.8 et 167.0 (C_{CONH}), 169.9 (C_{CO2Bn}); m/z (ESI^+) 263.1 (HRMS-ESI^+) 263.1035 ($[M+H]^+$ $C_{13}H_{15}N_2O_4$ calculated 263.1032).

4.4.13. Cyclo-[Gly-Lys(Z)] **4k**

Following the general procedure, pathway B, compound **4k** was isolated as a white crystal (35%), mp 199 °C, t_r = 1.412 min. δ_H (300 MHz, DMSO-d6; Me_4Si) δ 1.21–1.45 (m, 2H + 2H, $H_{CH*CH2CH2}$ + $H_{NHCH2CH2}$), 1.66 (m, 2H, H_{CH*CH2}), 2.96–3.00 (m, 2H, H_{NHCH2}), 3.69–3.76 (m, 2H + 1H, $H_{NHCH2CO}$ + H_{CH*}), 5.00 (s, 2H, H_{OCH2Ph}), 7.25 (s, 1H, H_{CH2NHZ}), 7.31–7.37 (m, 5H, H_{arom}), 7.99 (s, 1H, H_{CH2NH}), 8.16 (s, 1H, H_{CH*NH}); δ_C (75 MHz, DMSO-d6; Me_4Si) δ 21.3 (C_{CH*CH2}), 29.1 ($C_{CH2CH2CH2NHZ}$), 32.4 ($C_{CH2CH2NHZ}$), 40.1 (C_{CH2NHZ}), 44.2 (C_{CH2Gly}), 54.0 (C_{CH*}), 65.1 ($C_{CH2Phenyl}$), 127.1–137.2 (C_{arom}), 156.0 ($C_{NHCOOBn}$), 166.1 ($C_{COlactam}$), 168.0 ($C_{COlactam}$); m/z (ESI^+) 320.2 (HRMS-ESI^+) 320.1602 ($[M+H]^+$ $C_{16}H_{22}N_3O_4$ calculated 320.1610).

4.5. General experimental procedure for the synthesis of Bis-Boc DKP **6**

To a solution of DKP **4** (35.00 mmol) and di-*tert*-butyl dicarbonate Boc_2O (73.50 mmol) in dry DMF (50 mL) was slowly added DMAP (73.50 mmol). After stirring at room temperature under argon atmosphere for 1.5 h, the solution was diluted with AcOEt (300 mL) and then washed with 1.0 N $KHSO_4$ aqueous solution (200 mL). After drying on $MgSO_4$, the solvent was removed *in vacuo*. A rapid filtration on silica gel (CH_2Cl_2:AcOEt 90:10) afforded the activated DKP in 79–90% yield.

4.5.1. Bis-Boc Cyclo-[Gly-Val] **6b**

Following the general procedure, compound **6b** was isolated as white crystals (90%), mp 130 °C, t_r = 2.400 min. δ_H (300 MHz, $CDCl_3$; Me_4Si) δ 0.99 (d, 3H, J = 6.7 Hz, H_{CH-CH3}), 1.04 (d, 3H, J = 6.7 Hz, H_{CH-CH3}), 1.47 (s, 9H, H_{CCH3}), 1.48 (s, 9H, H_{CCH3}), 1.92–2.08 (m, 1H, $H_{CH(CH3)2}$), 4.08 (d, 1H, J = 18.6 Hz, H_{CH2}), 4.53 (d, 1H, J = 9.5 Hz, H_{CH*}), 4.67 (d, 1H, J = 18.6 Hz, H_{CH2}); δ_C (75 MHz, $CDCl_3$; Me_4Si) δ 19.4 (C_{CH3CH}), 19.5 (C_{CH3CH}), 27.9 (C_{CH3C}), 31.8 ($C_{CH(CH3)2}$), 49.1 (C_{CH2}), 65.4 (C_{CH*}), 84.8 ($C_{C(CH3)3}$), 84.9 ($C_{C(CH3)3}$), 149.9 ($C_{COurethane}$), 150.1 ($C_{COurethane}$), 164.9 ($C_{COlactam}$), 165.7 ($C_{COlactam}$); m/z (ESI^+) 357.2 (HRMS-ESI^+) 357.2043 ($[M+H]^+$ $C_{17}H_{29}N_2O_6$ calculated 357.2026).

4.5.2. Bis-Boc Cyclo-[Gly-AllylGly] **6f**

Following the general procedure, compound **6f** was isolated as white crystals (84%), mp 91 °C, t_r = 2.348 min. δ_H (300 MHz, $CDCl_3$; Me_4Si) δ 1.47 (s, 9H, $H_{C(CH3)3}$), 1.48 (s, 9H, $H_{C(CH3)3}$), 2.48–2.70 (m, 2H, $H_{CH2-CH=CH2}$), 4.09 (d, 1H, J = 18.4 Hz, H_{CH2Gly}), 4.64 (d, 1H, J = 18.4 Hz, H_{CH2Gly}), 4.85 (dd, 1H, J = 7.3 Hz, J = 6.4 Hz, H_{CH*}), 5.10–5.16 (m, 2H, $H_{CH=CH2}$), 5.64–5.83 (m, 1H, $H_{CH=CH2}$); δ_C (75 MHz, $CDCl_3$; Me_4Si) δ 27.5 ($C_{C(CH3)3}$), 27.6 ($C_{C(CH3)3}$), 37.0 ($C_{CH2-CH*}$), 48.5 (C_{CH2Gly}), 59.3 (C_{CH*}), 84.5 ($C_{C(CH3)3}$), 84.7 ($C_{C(CH3)3}$), 120.3 ($C_{CH2=CH}$), 130.7 ($C_{CH=CH2}$), 149.3 (C_{COBoc}), 149.4 (C_{COBoc}), 163.9 ($C_{COlactam}$), 165.8 ($C_{COlactam}$); m/z (ESI^+) 355.2 (HRMS-ESI^+) 355.1859 ($[M+H]^+$ $C_{17}H_{27}N_2O_6$ calculated 355.1869).

4.5.3. Bis-Boc Cyclo-[Gly-Thr(OBn)] **6i**

Following the general procedure, compound **6i** was isolated as white crystals (79%), mp 135 °C, t_r = 2.766 min. δ_H (300 MHz, $CDCl_3$; Me_4Si) δ 1.28 (d, 3H, J = 6.4 Hz, H_{CHCH3}), 1.54 (s, 9H, $H_{C(CH3)3}$), 1.55 (s, 9H, $H_{C(CH3)3}$), 4.19 (d, 1H, J = 18.1 Hz, H_{CH2Gly}), 4.30 (m, 1H, H_{CHOBn}), 4.34 (d, 1H, J = 11.1 Hz, H_{CH2Ph}), 4.39 (d, 1H, J = 18.1 Hz, H_{CH2Gly}), 4.60 (d, 1H, J = 11.1 Hz, H_{CH2Ph}), 5.00 (d, 1H, J = 2.7 Hz, $H_{COCH(N)CH}$), 7.19–7.33 (m, 5H, H_{arom}); δ_C (75 MHz, $CDCl_3$; Me_4Si) δ 14.7 (C_{CHCH3}), 27.0 ($C_{C(CH3)3}$), 49.2 (C_{CH2Gly}), 63.4 (C_{CH*}), 70.3 (C_{CH2Ph}), 76.9 (C_{CHOBn}), 83.7 ($C_{C(CH3)3}$), 83.8 ($C_{C(CH3)3}$), 126.8–136.4 (C_{arom}), 149.0 (C_{COBoc}), 149.4 (C_{COBoc}), 165.1 ($C_{COlactam}$), 166.8 ($C_{COlactam}$); m/z (ESI^+) 449.2 (HRMS-ESI^+) 449.2291 ($[M+H]^+$ $C_{23}H_{33}N_2O_7$ calculated 449.2288).

4.6. General experimental procedure for the TRAL/alkylation

To a stirred solution of **6** (7.00 mmol) in dry THF (30 mL), cooled at −78 °C under argon, was added dropwise a solution of LiHMDS 1.0 M in THF (7.00 mmol). After 45 min, the alkylating agent (7.00 mmol) was added. The reaction was left at room temperature overnight. The mixture was then diluted with AcOEt (20 mL) and washed with 0.1 N solution of HCl (10 mL). The organic layer was then dried on $MgSO_4$ and concentrated *in vacuo*. The crude mixture obtained was purified by column chromatography (petroleum ether/AcOEt), providing the desired compound.

*4.6.1. (3R,5R)-3-(tert-butoxycarbonylamino)-3-((E)-4-ethoxy-4-oxobut-2-enyl)-5-isopropyl-2,4-dioxopyrrolidine-1-carboxylic acid tert-butyl ester (**7c**)*

Following the general procedure, compound **7c** was isolated as a yellowish oil (42%), t_r = 2.824 min, δ_H (300 MHz, $CDCl_3$; Me_4Si) δ 0.95 (d, 3H, J = 6.9 Hz, $H_{(C\mathbf{H}3)2CH}$), 1.21 (d, 3H, J = 6.9 Hz, $H_{(C\mathbf{H}3)2CH}$), 1.30 (t, 3H, J = 7.2 Hz, $H_{C\mathbf{H}3-CH2}$), 1.37 (s, 9H, $H_{C(C\mathbf{H}3)3}$), 1.56 (s, 9H, $H_{C(C\mathbf{H}3)3}$), 2.42 (m, 1H, $H_{C\mathbf{H}(CH3)2}$), 2.58 (m, 2H, $H_{C\mathbf{H}2-CH=CH-CO2Et}$), 4.21 (q, 2H, J = 7.2 Hz, $H_{C\mathbf{H}2-CH3}$), 4.53 (d, 1H, J = 4.2 Hz, $H_{C\mathbf{H}*CH(CH3)2}$), 5.20 (sl, 1H, $H_{N\mathbf{H}Boc}$), 5.96 (d, 1H, J = 15.6 Hz, $H_{C\mathbf{H}CO2Et}$), 6.95 (m, 1H, $H_{C\mathbf{H}=CH-CO2Et}$); δ_C (75 MHz, $CDCl_3$; Me_4Si) δ 14.2 ($C_{C\mathbf{H}3-CH2}$), 18.0 ($C_{CH(C\mathbf{H}3)2}$), 19.2 ($C_{CH(C\mathbf{H}3)2}$), 27.9 ($C_{C(C\mathbf{H}3)3}$), 28.1 ($C_{C(C\mathbf{H}3)3}$), 30.2 ($C_{\mathbf{C}H(CH3)2}$), 34.1 (C_{CH2-CH}), 60.8 ($C_{CH2-CH3}$), 69.9 ($C_{\mathbf{C}H*}$), 77.2 (C_{quat*}), 81.8 ($C_{\mathbf{C}(CH3)3}$), 84.4 ($C_{\mathbf{C}(CH3)3}$), 127.3 ($C_{\mathbf{C}H-CO2Et}$), 138.2 ($C_{\mathbf{C}H=CH-CO2Et}$), 149.5 ($C_{\mathbf{C}OBoc}$), 154.8 ($C_{\mathbf{C}OBoc}$), 165.1 ($C_{\mathbf{C}Oester}$), 171.0 ($C_{\mathbf{C}Olactam}$), 204.9 ($C_{\mathbf{C}Oketone}$); *m/z* (ESI^+) 469.3 (HRMS-ESI^+) 469.2541 ($[M+H]^+$ $C_{23}H_{37}N_2O_8$ calculated 469.2550).

*4.6.2. (3R,5R)-3-(tert-butoxycarbonylamino)-5-isopropyl-3-((E)-3-methoxycarbonyl-allyl)-2,4-dioxo-pyrrolidine-1-carboxylic acid tert-butyl ester (**7d**)*

Following the general procedure, compound **7d** was isolated as a yellowish oil (49%), t_r = 2.696 min, δ_H (300 MHz, $CDCl_3$; Me_4Si) δ 0.97 (d, 3H, J = 6.9 Hz, $H_{(C\mathbf{H}3)2CH}$), 1.23 (d, 3H, J = 6.9 Hz, $H_{(C\mathbf{H}3)2CH}$), 1.38 (s, 9H, $H_{C(C\mathbf{H}3)3}$), 1.58 (s, 9H, $H_{C(C\mathbf{H}3)3}$), 2.44 (m, 1H, $H_{C\mathbf{H}(CH3)2}$), 2.60 (m, 2H, $H_{C\mathbf{H}2-CH=CH-CO2Me}$), 3.77 (s, 3H, $H_{CO2C\mathbf{H}3}$), 4.55 (d, 1H, J = 4.1 Hz, $H_{C\mathbf{H}*CH(CH3)2}$), 5.14 (s, 1H, $H_{N\mathbf{H}Boc}$), 5.98 (d, 1H, J = 15.7 Hz, $H_{C\mathbf{H}CO2Me}$), 6.96 (m, 1H, $H_{C\mathbf{H}=CH-CO2Me}$); δ_C (75 MHz, $CDCl_3$; Me_4Si) δ 18.0 ($C_{CH(C\mathbf{H}3)2}$), 19.2 ($C_{CH(C\mathbf{H}3)2}$), 28.0 ($C_{C(C\mathbf{H}3)3}$), 28.1 ($C_{C(C\mathbf{H}3)3}$), 30.2 ($C_{\mathbf{C}H(CH3)2}$), 34.1 ($C_{\mathbf{C}H2-CH}$), 51.8 ($C_{CO2\mathbf{C}H3}$), 69.9 ($C_{\mathbf{C}H*}$), 77.2 (C_{quat*}), 81.9 ($C_{\mathbf{C}(CH3)3}$), 84.4 ($C_{\mathbf{C}(CH3)3}$), 126.9 ($C_{\mathbf{C}H-CO2Me}$), 138.5 ($C_{\mathbf{C}H=CH-CO2Me}$), 149.5 ($C_{\mathbf{C}OBoc}$), 154.7 ($C_{\mathbf{C}OBoc}$), 165.5 ($C_{\mathbf{C}Oester}$), 170.9 ($C_{\mathbf{C}Olactam}$), 204.9 ($C_{\mathbf{C}Oketone}$); *m/z* (ESI^+) 455.2 (HRMS-ESI^+) 455.2392 ($[M+H]^+$ $C_{22}H_{35}N_2O_8$ calculated 455.2393).

*4.6.3. 5-Allyl-3-tert-butoxycarbonylamino-3-(3-methoxycarbonyl-allyl)-2,4-dioxo-pyrrolidine-1-carboxylic acid tert-butyl ester (**7j**)*

Following the general procedure, compound **7j** was isolated as a yellowish oil (35%), t_r = 2.591 min, δ_H (300 MHz, $CDCl_3$; Me_4Si) δ 1.23 (s, 9H, $H_{C(C\mathbf{H}3)3}$), 1.35 (s, 9H, $H_{C(C\mathbf{H}3)3}$), 2.55 (m, 2H, $H_{C\mathbf{H}2CH=CH}$), 2.69–2.90 (m, 2H, $H_{CH*C\mathbf{H}2}$), 3.74 (s, 3H, $H_{CO2C\mathbf{H}3}$), 4.65 (dd, 1H, J = 3.1 Hz, J = 6.6 Hz, $H_{C\mathbf{H}*}$), 5.10–5.22 (m, 3H, $H_{CH=C\mathbf{H}2}$ + $H_{N\mathbf{H}Boc}$), 5.65 (m, 1H, $H_{C\mathbf{H}=CH2}$), 5.93 (d, 1H, J = 15.6 Hz, $H_{CH2CH=C\mathbf{H}CO2Me}$), 6.88 (m, 1H, $H_{CH2C\mathbf{H}=CHCO2Me}$); δ_C (75 MHz, $CDCl_3$; Me_4Si) δ 28.0 ($C_{C(C\mathbf{H}3)3}$), 28.1 ($C_{C(C\mathbf{H}3)3}$), 34.2 & 34.3 ($C_{CH*\mathbf{C}H2}$ & $C_{\mathbf{C}H2CH=CH}$), 51.9 ($C_{CO2\mathbf{C}H3}$), 60.7 (C_{quat}), 65.1 ($C_{\mathbf{C}H*}$), 82.0 ($C_{\mathbf{C}(CH3)3}$), 84.5 ($C_{\mathbf{C}(CH3)3}$), 120.8 ($C_{CH=\mathbf{C}H2}$), 127.0 ($C_{\mathbf{C}HCO2Me}$), 131.5 ($C_{\mathbf{C}H2=CH}$), 138.4 ($C_{\mathbf{C}H=CHCO2Me}$), 149.1 ($C_{\mathbf{C}OBoc}$), 154.8 ($C_{\mathbf{C}OBoc}$), 165.5 ($C_{\mathbf{C}Oester}$), 170.7 ($C_{\mathbf{C}Olactam}$), 204.6 ($C_{\mathbf{C}Oketone}$); *m/z* (ESI^+) 453.2 (HRMS-ESI^+) 453.2240 ($[M+H]^+$ $C_{22}H_{33}N_2O_8$ calculated 453.2237).

*4.6.4. 5-(1-Benzyloxy-ethyl)-3-tert-butoxycarbonylamino-2,4-dioxo-3-prop-2-ynyl-pyrrolidine-1-carboxylic acid tert-butyl ester (**7n**)*

Following the general procedure, compound **7n** was isolated as a colorless oil (30%), t_r = 2.840 min, δ_H (300 MHz, $CDCl_3$; Me_4Si) δ 1.29 (d, 3H, J = 6.6 Hz, $H_{CHC\mathbf{H}3}$), 1.34 (s, 9H, $H_{C(C\mathbf{H}3)3}$), 1.54 (s, 9H, $H_{C(C\mathbf{H}3)3}$), 2.17 (t, 1H, J = 2.6 Hz, $H_{C\equiv C\mathbf{H}}$), 2.51 (dd, 1H, J = 2.6 Hz, J = 17.5 Hz, $H_{C\mathbf{H}2C\equiv CH}$), 2.62 (dd, 1H, J = 2.6 Hz, J = 17.5 Hz, $H_{C\mathbf{H}2C\equiv CH}$), 4.10 (m, 1H, $H_{C\mathbf{H}CH3}$), 4.27 (d, 1H, J = 11.0 Hz, $H_{C\mathbf{H}2C6H5}$), 4.52 (d, 1H, J = 11.0 Hz, $H_{C\mathbf{H}2C6H5}$), 4.72 (d, 1H, J = 2.7 Hz, H_{CH*}), 5.52 (br s, 1H, H_{NHBoc}), 7.21–7.34 (m, 5H, $H_{C6\mathbf{H}5}$); δ_C (75 MHz, $CDCl_3$; Me_4Si) δ 16.7 ($C_{CH\mathbf{C}H3}$), 22.0 ($C_{\mathbf{C}H2-C\equiv CH}$), 27.9 ($C_{C(C\mathbf{H}3)3}$), 28.1 ($C_{C(C\mathbf{H}3)3}$), 69.2, 71.2, 73.6, 75.0, 77.2, 81.7 ($C_{\mathbf{C}(CH3)3}$), 84.3 ($C_{\mathbf{C}(CH3)3}$), 128.2–136.7 ($C_{\mathbf{C}6H5}$), 150.3 ($C_{\mathbf{C}OBoc}$), 155.1 ($C_{\mathbf{C}OBoc}$), 171.0 ($C_{\mathbf{C}Olactam}$), 204.2 ($C_{\mathbf{C}Oketone}$); *m/z* (ESI^+) 487.2 (HRMS-ESI^+) 487.2448 ($[M+H]^+$ $C_{26}H_{35}N_2O_7$ calculated 487.2444).

*4.6.5. (3R,5R)-4-(3-tert-Butoxycarbonylamino-5-isopropyl-2,4-dioxo-pyrrolidin-3-yl)-but-2-enoic acid ethyl ester **9c***

To a solution of (3*R*,5*R*)-3-(tert-butoxycarbonylamino)-3-((*E*)-4-ethoxy-4-oxobut-2-enyl)-5-isopropyl-2,4-dioxopyrrolidine-1-carboxylic acid tert-butyl ester **7c** (100 mg, 0.21 mmol) in dichloromethane (5 mL) was added dropwise trifluoroacetic acid (0.15 mL) at 0 °C. The reaction mixture was stirred during 2.5 h at 0 °C. The reaction media was then evaporated to dryness, and the remaining TFA was coevaporated with toluene to afford **9c** (71 mg, 90%) as a yellowish oil, t_r = 2.068 min. δ_H (300 MHz, $CDCl_3$; Me_4Si) δ 0.91 (d, 3H, J = 6.7 Hz, $H_{(C\mathbf{H}3)2CH}$), 1.02 (d, 3H, J = 6.7 Hz, $H_{(C\mathbf{H}3)2CH}$), 1.23 (t, 3H, J = 7.1 Hz, $H_{C\mathbf{H}3-CH2}$), 1.32 (s, 9H, $H_{C(C\mathbf{H}3)3}$), 2.07 (m, 1H, $H_{C\mathbf{H}(CH3)2}$), 2.49 (m, 2H, $H_{C\mathbf{H}2-CH=CH-CO2Et}$), 4.01 (d, 1H, $H_{C\mathbf{H}*}$), 4.14 (q, 2H, J = 7.1 Hz, $H_{C\mathbf{H}2-CH3}$), 5.10 (sl, 1H, $H_{N\mathbf{H}Boc}$), 5.89 (d, 1H, J = 15.6 Hz, $H_{C\mathbf{H}-CO2Et}$), 6.47 (sl, 1H, $H_{N\mathbf{H}}$), 6.88 (m, 1H, $H_{C\mathbf{H}=CH-CO2Et}$); δ_C (75 MHz, $CDCl_3$; Me_4Si) δ 14.2 ($C_{C\mathbf{H}3-CH2}$), 18.4 ($C_{CH(C\mathbf{H}3)2}$), 19.3 ($C_{CH(C\mathbf{H}3)2}$), 28.1 ($C_{C(C\mathbf{H}3)3}$), 30.4 ($C_{\mathbf{C}H(CH3)2}$), 35.2 ($C_{\mathbf{C}H2-CH=CH-CO2Et}$), 59.6 ($C_{\mathbf{C}H2-CH3}$), 60.7 ($C_{\mathbf{C}H*}$), 67.5 ($C_{quat*}$), 81.7 ($C_{\mathbf{C}(CH3)3}$), 127.2 ($C_{\mathbf{C}H=CH-CO2Et}$), 138.6 ($C_{\mathbf{C}H-CO2Et}$), 154.8 ($C_{\mathbf{C}OBoc}$), 165.2 ($C_{\mathbf{C}Oester}$), 173.0 ($C_{\mathbf{C}Olactam}$), 207.1 ($C_{\mathbf{C}Oketone}$); *m/z* ($ESI^+$) 369.2 (HRMS-$ESI^+$) 369.2024 ($[M+H]^+$ $C_{18}H_{29}N_2O_6$ calculated 369.2026).

*4.6.6. TFA salt of (3R,5R)-4-(3-Amino-5-isopropyl-2,4-dioxo-pyrrolidin-3-yl)-but-2-enoic acid ethyl ester **10c***

To a solution of (3*R*,5*R*)-3-(tert-butoxycarbonylamino)-3-((*E*)-4-ethoxy-4-oxobut-2-enyl)-5-isopropyl-2,4-dioxopyrrolidine-1-carboxylic acid tert-butyl ester **7c** (100 mg, 0.21 mmol) in dichloromethane (5 mL) was added dropwise trifluoroacetic acid (0.5 mL) at room temperature. The reaction mixture was stirred during 2 h at room temperature. Then, the reaction media was evaporated to dryness, and the remaining TFA was coevaporated with toluene to afford **10c** (82 mg, 100%) as a brownish oil, t_r = 1.284 min. δ_H (300 MHz, $CDCl_3$; Me_4Si) δ 0.89 (d, 3H, J = 6.7 Hz, $H_{CH(C\mathbf{H}3)2}$), 1.00 (d, 3H, J = 6.7 Hz, $H_{CH(C\mathbf{H}3)2}$), 1.21 (t, 3H, J = 7.1 Hz, $H_{C\mathbf{H}3-CH2}$), 1.65 (sl, 2H,, $H_{N\mathbf{H}2}$), 2.04 (m, 1H, $H_{C\mathbf{H}(CH3)2}$), 2.51 (m, 2H, $H_{C\mathbf{H}2-CH=CH-CO2Et}$), 3.82 (d, 1H, J = 5.4 Hz, $H_{C\mathbf{H}*}$), 4.11 (q, 2H, J = 7.1 Hz, $H_{C\mathbf{H}2-CH3}$), 5.85 (d, 1H, J = 15.6 Hz, $H_{C\mathbf{H}-CO2Et}$), 6.82 (m, 1H, $H_{C\mathbf{H}=CH-CO2Et}$), 7.47 (sl, 1H, $H_{N\mathbf{H}}$); δ_C (75 MHz, $CDCl_3$; Me_4Si) δ 13.6 ($C_{C\mathbf{H}3-CH2}$), 17.5 ($C_{CH(C\mathbf{H}3)2}$), 18.5 ($C_{CH(C\mathbf{H}3)2}$), 30.0 ($C_{\mathbf{C}H(CH3)2}$), 37.8 ($C_{\mathbf{C}H2-CH=CH-CO2Et}$), 59.2 ($C_{\mathbf{C}H2-CH3}$), 59.9 ($C_{\mathbf{C}H*}$), 65.9 ($C_{quat}$), 125.9 ($C_{\mathbf{C}H=CH-CO2Et}$), 139.3 ($C_{\mathbf{C}H-CO2Et}$), 164.9 ($C_{\mathbf{C}Oester}$), 174.1 ($C_{\mathbf{C}Olactam}$), 208.9 ($C_{\mathbf{C}Oketone}$); *m/z* ($ESI^+$) 269.1 (HRMS-$ESI^+$) 269.1497 ($[M+H]^+$ $C_{13}H_{21}N_2O_4$ calculated 269.1501).

*4.6.7. (3R,5R)-3-Isopropyl-2,6-diaza-spiro[4.5]decane-1,4,7-trione **11***

The TFA salt of (3*R*,5*R*)-4-(3-Amino-5-isopropyl-2,4-dioxo-pyrrolidin-3-yl)-but-2-enoic acid ethyl ester **10c** (200 mg, 0.52 mmol) was dissolved in ethanol and reduced via catalytic hydrogenation with 10% Pd/C at room temperature for 46 h. The catalyst was filtered off trough Celite, and the solvent was removed *in vacuo* to give a tan oil. This material was then redissolved in EtOH and heated with a microwave oven (Biotage Initiator Microwave Synthesizer Producing controlled radiation at 2450 MHz and using fixed hold-time) at 150 °C for 1 h. After evaporation, the crude residue was purified on a RP-18 column (H_2O/EtOH 100:0 ≫ 65:35 in 35 min) to afford **11** (95 mg, 81%), as a yellow oil, t_r = 1.163 min. δ_H (300 MHz, DMSO-d6; Me_4Si) δ 0.90 (d, 3H, J = 6.7 Hz, $H_{CH(C\mathbf{H}3)2}$), 0.96 (d, 3H, J = 6.7 Hz, $H_{CH(C\mathbf{H}3)2}$), 1.18–2.22 (m, 7H, $H_{C\mathbf{H}2CH2CH2CO}$ + $H_{CH2C\mathbf{H}2CH2CO}$ + $H_{CH2CH2C\mathbf{H}2CO}$ + $H_{C\mathbf{H}(CH3)2}$), 3.67 (d, 1H, J = 6.2 Hz, $H_{C\mathbf{H}*}$), 7.50 (br s, 1H, $H_{N\mathbf{H}}$), 8.81 (br s, 1H, $H_{N\mathbf{H}}$); δ_C (75 MHz, DMSO-d6; Me_4Si) δ 16.4 ($C_{CH2\mathbf{C}H2CH2CO}$), 18.5 ($C_{CH(C\mathbf{H}3)2}$), 18.8 ($C_{CH(C\mathbf{H}3)2}$), 27.9/30.7/30.9 ($C_{\mathbf{C}H2CH2CH2CO}$ + $C_{CH2CH2\mathbf{C}H2CO}$ + $C_{\mathbf{C}H(CH3)2}$),

60.8 (C_{quat}), 66.0 (C_{CH*}), 171.0 ($C_{COlactam}$), 172.8 ($C_{COlactam}$), 210.4 ($C_{COketone}$); *m/z* (ESI$^+$) 225.1 (HRMS-ESI$^+$) 225.1231 ([M + H]$^+$ $C_{11}H_{17}N_2O_3$ calculated 225.1239).

4.7. Biological assays

Cell culture conditions and proliferation assay: the human breast cancer cell line SKBR3 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.1% Sodium pyruvate. Compounds stock solutions were dissolved in dimethyl sulfoxide (10 mM), aliquoted and stored at −20 °C. In anti-proliferative assays (each condition was performed in triplicate), compounds were assayed for their growth inhibiting activity using the CellTiter-Glo™ Luminescent Cell Viability Assay as described by the manufacturer (Promega Corporation). Briefly, 10,000 cells were plated onto 96 well-plates (white with clear bottom (3610, Corning Costar) in 100 μL media per well and were allowed to grow overnight before assay. Compounds were added at different concentrations (varying from 100 to 0.1 μM) to each well and cell cultures were incubated for 72 h. Vehicle (DMSO) was used as control and all compounds were tested in constant percentage of DMSO (1%). After addition of 50 μL CellTiter-Glo™, luminescence was measured using a Centro luminometer (Berthold).

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