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Examination of the active secondary structure of the peptide 101.10, an allosteric modulator of the interleukin-1 receptor, by positional scanning using β-amino γ-lactams

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The relationship between the conformation and biological activity of the peptide allosteric modulator of the interleukin-1 receptor 101.10 (D-Arg-D-Tyr-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2) has been studied using (*R*)- and (*S*)-Bgl residues. Twelve Bgl peptides were synthesized using (*R*)- and (*S*)-cyclic sulfamidate reagents derived from L- and D-aspartic acid in an optimized Fmoc-compatible protocol for efficient lactam installment onto the supported peptide resin. Examination of these (*R*)- and (*S*)-Bgl 101.10 analogs for their potential to inhibit IL-1β-induced thymocyte cell proliferation using a novel fluorescence assay revealed that certain analogs exhibited retained and improved potency relative to the parent peptide 101.10. In light of previous reports that Bgl residues may stabilize type II′ β-turn-like conformations in peptides, CD spectroscopy was performed on selected compounds to identify secondary structure necessary for peptide biological activity. Results indicate that the presence of a fold about the central residues of the parent peptide may be important for activity.

Keywords: Freidinger lactams; β-amino γ-lactam; lactam scanning; interleukin-1 receptor; 101.10; CD; peptide mimic; β-turn; conformational analysis; solid-phase peptide synthesis

Introduction

Among the various cytokines involved in inflammatory conditions, evidence reveals the dominant role for interleukin-1β (IL-1β), a 17 kDa pleiotropic cytokine, in numerous chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, osteoarthritis and psoriasis [1,2]. Interleukin-1β interplays with important mediators of inflammation, including TNF, IL-18 and IL-6 [3]. The biological effects of IL-1β occur upon interaction with the IL-1RI, which is composed of a ligand-binding and a signaling subunit (named the IL-1RAcP), or with the IL-1RII, which acts as a decoy receptor [1]. Stimulation of IL-1RI by IL-1β leads to the activation of downstream signaling mediators, notably PGE_2 and NF-κB, eliciting hyperthermia and proinflammatory effects [4].

The recombinant protein of the natural IL-1ra (17.5 kDa; Kineret™) has been demonstrated to compete with the latter for receptor binding and is presently the only clinically effective approach for interfering specifically with IL-1β action [5]. Daily subcutaneous administration of IL-1ra is, however, associated with limited efficacy [6] and undesirable adverse effects such as injection-site reactions, increased susceptibility to gram-positive bacterial infections and immunogenicity [7]. Although small organic molecules which influence IL-1β activity, such as the diaryl sulphonyl urea CP-424,174, an IL-1β post-translational blocker, and pyridine carboxylate CJ-14,877, are under development (Figure 1), the application of such drug candidates may be limited because of their stimulus-dependent mechanism of action and lower potency (μM range). On the other hand, peptides and small proteins, which can mimic specific regions of the protein's receptor sequence, may interfere with its mechanism of action by inhibiting normal protein folding and interactions with other subunits [8–12]. For example, a recombinant protein of the major extracellular portion of the IL-1RAcP has exhibited *in vivo* efficacy in a model of autoimmune arthritis [13].

Employing crystallographic data and computational analysis (i.e. modeling with PROSITE and ProtScale) [14–16] to evaluate

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Abbreviations used: *AcOH, acetic acid; Agl, α-amino γ-lactam; Bgl, β-amino γ-lactam; CD36, cluster of differentiation 36; DIEA,* N,N-*diisopropylethylamine; GHRP-6, growth hormone releasing peptide-6; GHS-R1a, growth hormone secretagog receptor 1a; GM-CSF, granulocyte macrophage colony stimulating factor; IL-1ra, IL-1 receptor antagonist; IL-1RAcP, IL-1R accessory protein; IL-1RI, IL-1 receptor type I; NF-κB, nuclear factor-κB; PGE_2, prostaglandin E_2; PPII, poly proline type II helix; RP-HPLC, reverse phase high performance liquid chromatography; RPMI 1640, Roswell Park Memorial Institute medium 1640; TES, triethyl silane; TNF, tumor necrosis factor.*

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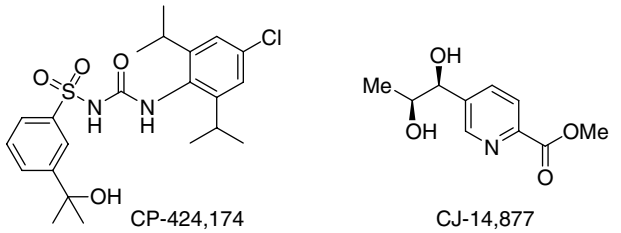

Figure 1. Small molecules which influence IL-1β activity.

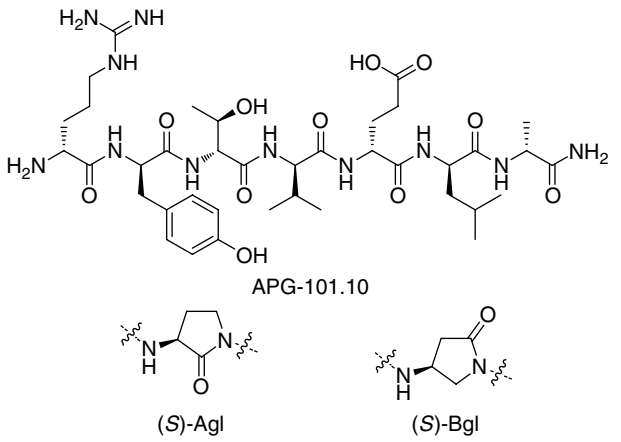

Figure 2. 101.10, (*S*)-Agl and (*S*)-Bgl residues.

hydrophobicity, flexibility and homology domains [17,18], peptide sequences were previously selected from the extracellular loops and inter-domain regions of the IL-1RAcP protein, and examined for activity against IL-1β-induced effects [19]. Guided by their efficacy and potency in inhibiting thymocyte proliferation and PGE_2 synthesis, a lead candidate was developed featuring an all D-amino acid sequence [101.10, rytvela (D-amino acids are written in small letters), Figure 2], which exhibited an inhibitory potency (IC_{50}) of 2 nM and an antiproliferative efficacy (E_{max}) of >90% [19]. In a further study involving models of hyperthermia, inflammatory bowel disease and contact dermatitis, 101.10 proved superior to corticosteroids and IL-1ra [19]. 101.10 was characterized as a noncompetitive antagonist, which exhibited properties of an allosteric negative modulator, such as functional selectivity, blockade of certain IL-1β pathways without affecting others and modulation of ligand-binding affinity. 101.10 did not bind to IL-1RI deficient cells and was ineffective in IL-1RI knockout mice [19].

The importance of side chain interactions for biological activity was demonstrated in an earlier structure-activity analysis of a longer peptide precursor of 101.10 (aprytvela) [20]. Measuring inhibition of PGE_2 synthesis, a systematic scan demonstrated that replacement of D-Ala at each residue reduced activity: exchange at D-Tyr caused a 20% reduction; at D-Arg and D-Thr caused >50% reduction and at D-Val, D-Glu and D-Leu activity was abolished [20]. This preliminary D-Ala scan analysis foretold that attempts to change the peptide backbone conformation may lead to activity loss, because of removal of the side chain functionality.

Exploring the active conformation of 101.10, we have previously described the synthesis of analogs bearing one and two Agl residues (Figure 2) [21,22]. As first demonstrated in pioneering studies on luteinizing-hormone releasing-hormone, (*S*)-Agl can induce peptides to adopt a type II′ β-turn conformation [23]. In head-to-head comparisons with the parent peptide, [(*R*)-Agl^1] 101.10 in which the D-Arg^1 position was substituted by the (*R*)-Agl exhibited a 2.2-fold increase in efficacy in reducing the effect of IL-1β on thymocyte proliferation. Although replacement of the D-Thr^3 residue by (*R*)-Agl failed to improve efficacy, analogs possessing one (*R*)-Agl residue, respectively at either the D-Tyr^2, D-Val^4, D-Glu^5 or D-Leu^6 position, demonstrated similar efficacy compared to 101.10 [21].

β-Amino γ-lactam (Bgl) residues (Figure 2) have also been suggested to stabilize β-turn-like conformations in peptides [24]. The complementary application of Agl and Bgl residues may thus reveal information concerning the biologically active fold of the parent peptide. For example, the replacement of the Leu residue of the dopamine receptor modulator Pro-Leu-Gly-NH_2, respectively, by Agl and Bgl residues gave analogs that were 8 and 37% more potent than the parent tripeptide at 1 nM [25,26]. By conducting a systematic scan of the GHRP-6 using both (*R*)- and (*S*)-Agl and Bgl residues, we have previously found that replacement of the Ala^3 residue gave analogs that were selective for the CD36 receptor relative to the GHS-R1a, demonstrating the importance of turn geometry around the central Ala^3 residue for activity at the CD36 scavenger receptor [27].

We now report systematic scans of the 101.10 sequence using (*S*)- and (*R*)-Bgl residues. Twelve new 101.10 analogs, (*R*)- and (*S*)-**1**–**6** were examined for their potential to inhibit IL-1β-induced thymocyte proliferation. Furthermore, the most efficacious analogs were examined by CD spectroscopy and compared with their diastereomeric Bgl counterparts. These preliminary results have provided more potent analogs than the parent peptide as well as insight into the conformational preferences for biological activity.

Materials and Methods

General Methods

Sulfamidates (*S*)- and (*R*)-**7** were synthesized, respectively, from L- and D-aspartic acid as previously described [21]. Rink amide MBHA (4-methylbenzhydrylamine) resin (100–200 mesh, 0.7 mmol/g loading) was purchased from Novabiochem® (EMD Bioscience Inc., San Diego, CA, USA). Fmoc amino acids [i.e. Fmoc-D-Ala, Fmoc-D-Leu, Fmoc-D-Glu(OtBu), Fmoc-D-Val, Fmoc-D-Thr(OtBu), Fmoc-D-Pro, Fmoc-D-Tyr(tBu), and Fmoc-D-Arg(*N*-Pbf)] were purchased from Novabiochem® (EMD Bioscience Inc.) or GL Biochem Ltd. (Shangai, China). *O*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU) was purchased from GL Biochem (Shangai, China). Anhydrous solvents (THF and DMF) were obtained from a Seca Solvent Filtration System (GlassContour™ Laguna Beach, CA, USA). DIEA (Aldrich®, St Louis, MO, USA) was distilled first from ninhydrin, then from calcium hydride. Peptide couplings and Fmoc removal were qualitatively monitored by Kaiser Test [28]. Microwave-assisted reactions were performed in a Biotage Initiator microwave apparatus (Biotage AB, Uppsala, Sweden) in Biotage 2 ml V-shaped glass reaction vessels containing a 10-mm triangular magnetic stirrer bar and sealed with a septum cap. Alkylation and lactam formation reactions were monitored by reverse phase high performance liquid chromatography-mass spectrometry (RP-HPLC-MS) [UV: λ = 214 nm, 0–60% methyl cyanide (MeCN) in H_2O, 0.1% formic acid (FA) over 4 min, 0.5 ml/min, Gemini™ 5u C18 110A, 50 × 4.6 mm, 5 µm]. Analytical RP-HPLC analyses of crude and purified peptides were performed on a Gemini™ 5u C18 110A column (Phenomenex® Inc., Torrance,

CA, USA, 150 × 4.6 mm, 5 µm, column A) or a Synergi™ 4u Polar RP80A column (Phenomenex® Inc., 150 × 4.6 mm, 4 µm, column B) with a flow rate of 0.5 ml/min using a linear gradient of acetonitrile (0.1% FA) in water (0.1% FA). Retention times (t_R) from analytical RP-HPLC are reported in minutes, UV absorption was recorded at $\lambda = 214$ nm. Peptides were purified with a Gemini™ 5u C18 110A column (Phenomenex® Inc., 250 × 21.2 mm, 5 µm, column C) or a Synergi™ 4u Polar RP80A Axial Packed column (Phenomenex® Inc., 100 × 21.2 mm, 4 µm, column D) using a specified linear gradient of acetonitrile (0.1% FA), in water (0.1% FA), with a flow rate of 10.6 ml/min.

Peptide Synthesis and Bgl Installment Protocols

Resin swelling, Fmoc deprotection and washings

A 6-ml plastic filtration tube with a polyethylene filter was charged with Rink MBHA resin (300 mg, 0.7 mmol/g) and DMF (4 ml). The tube was sealed and shaken for 0.5 h. The resin was then filtered and taken up in freshly prepared 20% piperidine in DMF solution (4 ml), shaken for 30 min, filtered, retreated with 20% piperidine/DMF solution (4 ml) and shaken for 30 min. The resin was washed by successive agitations for 1 min and filtered from DMF (3 × 4 ml), methanol (MeOH) (3 × 4 ml) and DCM (3 × 4 ml). A positive Kaiser color test indicated qualitatively the presence of free amine.

Amino acid coupling

The amine resin in a 6-ml plastic filtration tube with a polyethylene filter was first swollen in DMF as described above. Meanwhile, a solution of *N*-(Fmoc)amino acid (Fmoc-Xaa, 3 equiv.) and HBTU (3 equiv.) in DMF (4 ml) was stirred for 10 min in a small sample vial, to which DIEA (6 equiv.) was added. The mixture was stirred for 0.5 min and then added to the resin. The reaction mixture was shaken for 1 h with Fmoc-D-Ala, 3 h with Fmoc-D-Val and 4 h with Fmoc-D-Leu, Fmoc-D-Glu(^{t}Bu), Fmoc-D-Thr(^{t}Bu), Fmoc-D-Tyr(^{t}Bu) and Fmoc-D-Arg(Pbf), respectively, at room temperature. The resin was filtered and washed by shaking for 1 min with DMF (3 × 4 ml), MeOH (3 × 4 ml) and DCM (3 × 4 ml). A negative Kaiser test response indicated completion of the reaction. The resin was then dried *in vacuo*.

Bgl installation by peptide alkylation with sulfamidate **7** *and lactam formation*

After Fmoc deprotection and washings, peptide resin **8** was transferred into a 2 ml V-shaped glass microwave vessel and treated with a solution of cyclic sulfamidate **7** (5 equiv.) and DIEA (1 equiv.) in THF (2 ml). The reactor was sealed and heated at 60 °C under microwave irradiation for 1.5 h. The resulting sulfamate resin **9** was transferred into a 6 ml syringe tube, washed with DMF (3 × 4 ml), MeOH (3 × 4 ml) and DCM (3 × 4 ml), treated with three 30-min cycles of 2% acetic acid (AcOH) in DMSO (4 ml) followed by washing with MeCN (4 ml) for 5 min. A 2 ml V-shaped glass microwave vessel fitted with a triangular stirring bar was then charged with the resulting resin **10**, treated with a DMSO : H_2O : AcOH 75 : 23 : 2 mixture (2 ml), sealed and heated at 100 °C under microwave irradiation for ≥4 h (Table 1). The resulting lactam resin **11** was transferred from the microwave vessels into a 6-ml plastic filtration tube fitted with a polyethylene frit and washed sequentially with DMF (3 × 4 ml), MeOH (3 × 4 ml) and DCM (3 × 4 ml), and dried *in vacuo*. Conversions after the alkylation and lactam forming steps were monitored by RP-HPLC-MS analysis of material cleaved from a sample of resin (3 mg) using a TFA/H_2O/TES cocktail (1 ml, 95/2.5/2.5, v/v/v) for 1 h.

Resin capping and peptide completion

The resin **11** in a 6-ml plastic filtration tube was swollen in DMF, suspended in a solution of acetic anhydride (5 equiv.) and DIEA (10 equiv.) in DMF (4 ml) and shaken for 1 h. The resin was filtered and washed sequentially with DMF (3 × 4 ml), MeOH (3 × 4 ml) and DCM (3 × 4 ml). The *N*-terminal Fmoc group was removed and the peptide synthesis completed as described above.

Table 1. Crude purities and yields of >99% pure[a] Bgl peptide analogs of 101.10 **1**–**6**

Bgl-peptide[b]	Crude purity %[c] [lactamization time (h)]	Yield %[d]	HRMS	
			m/z (calcd)	*m/z* (obsd)
(*R*)-**1** (***R***)-**Bgl**-D-Tyr-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2	71 (4)	22	777.4141	777.4145
(*R*)-**2** D-Arg-(***R***)-**Bgl**-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2	87 (6)	24	770.4519	770.4521
(*R*)-**3** D-Arg-D-Tyr-(***R***)-**Bgl**-D-Val-D-Glu-D-Leu-D-Ala-NH_2	86 (4)	23	832.4676	832.4677
(*R*)-**4** D-Arg-D-Tyr-D-Thr-(***R***)-**Bgl**-D-Glu-D-Leu-D-Ala-NH_2	79 (7)	17	833.4468	833.4470
(*R*)-**5** D-Arg-D-Tyr-D-Thr-D-Val-(***R***)-**Bgl**-D-Leu-D-Ala-NH_2	87 (4)	7	826.4546[e]	826.4544[e]
(*R*)-**6** D-Arg-D-Tyr-D-Thr-D-Val-D-Glu-(***R***)-**Bgl**-D-Ala-NH_2	50 (4)	14	820.4312	820.4313
(*S*)-**1** (***S***)-**Bgl**-D-Tyr-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2	70 (10)	13	777.4141	777.4138
(*S*)-**2** D-Arg-(***S***)-**Bgl**-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2	82 (10)	16	770.4519	770.4518
(*S*)-**3** D-Arg-D-Tyr-(***S***)-**Bgl**-D-Val-D-Glu-D-Leu-D-Ala-NH_2	60 (8)	17	832.4676	832.4674
(*S*)-**4** D-Arg-D-Tyr-D-Thr-(***S***)-**Bgl**-D-Glu-D-Leu-D-Ala-NH_2	87 (7)	14	833.4468	833.4469
(*S*)-**5** D-Arg-D-Tyr-D-Thr-D-Val-(***S***)-**Bgl**-D-Leu-D-Ala-NH_2	90 (4)	13	803.4726	803.4724
(*S*)-**6** D-Arg-D-Tyr-D-Thr-D-Val-D-Glu-(***S***)-**Bgl**-D-Ala-NH_2	96 (4)	11	820.4131	820.4136

[a] RP-HPLC purity at 214 nm of the purified peptide (see Materials and Methods).
[b] Bold lettering indicates lactam residues.
[c] RP-HPLC purity at 214 nm of the crude peptide (see Materials and Methods).
[d] Yields after purification by RP-HPLC are based on Fmoc loading test for Rink resin.
[e] The cation $[M + Na]^+$ was observed, theoretical mass was calculated accordingly.

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Peptide cleavage

The resin was swollen in DMF and the *N*-terminal Fmoc group was cleaved as described above. The resin was filtered and washed by shaking for 1 min each and filtering from DMF (3 × 4 ml), MeOH (3 × 4 ml) and DCM (3 × 4 ml). A positive Kaiser color test indicated qualitatively the presence of free amine. The peptide was then cleaved from the resin by shaking in TFA/H_2O/TES (4 ml, 95/2.5/2.5, v/v/v) for 3 h. The resin was filtered, washed with TFA (3 × 4 ml) and the combined filtrate and washings were concentrated *in vacuo*. The resulting residue was dissolved in a minimum volume of TFA (~1 ml), transferred to a centrifuge tube, precipitated by the addition of ice-cold diethyl ether (40 ml) and centrifuged. The diethyl ether was carefully decanted from the tube, and the precipitated peptide was washed twice with cold diethyl ether. The resulting white solid was dissolved in water (10 ml) and freeze-dried to give white foam that was purified by preparative RP-HPLC, using the specified conditions.

(*R*)-Bgl-D-Tyr-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2 (*R*)-1

(*R*)-Bgl-D-Tyr-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2 (*R*)-**1** was prepared on a 0.072 mmol scale, in a syringe tube following the optimized protocol as described above, using microwave-assisted annulation over 4 h, to give the desired lactam peptide TFA salt (*R*)-**1** (34 mg) in 71% crude purity as determined by analytical RP-HPLC (column B, UV: λ = 214 nm, 0–80% MeCN in H_2O, 0.1% FA, 30-min gradient). Purification was carried out by preparative RP-HPLC (column D, 10–30% MeCN in H_2O, 0.1% FA, 30-min gradient) to give the desired FA salt (*R*)-**1** (13 mg, 22%) as a white fluffy solid. The purified product was analyzed by analytical RP-HPLC (column B, UV: λ = 214 nm) using both MeCN t_R 11.8 (5–60% MeCN in H_2O, 0.1% FA, 25-min gradient) and MeOH t_R 16.41 (0–80% MeOH in H_2O, 0.1% FA, 25-min gradient) and revealed to be of >99% purity. HRMS calcd *m/z* for $C_{36}H_{57}O_{11}N_8$ $[M + H]^+$ 777.4141, found 777.4145.

D-Arg-(*R*)-Bgl-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2 (*R*)-2

D-Arg-(*R*)-Bgl-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2 (*R*)-**2** was prepared on a 0.078 mmol scale, in a syringe tube following the optimized protocol as described above, using microwave-assisted annulation over 6 h, to give the desired lactam peptide TFA salt (*R*)-**2** (35 mg) in 87% crude purity as determined by analytical RP-HPLC (column A, UV: λ = 214 nm, 0–20% MeCN in H_2O, 0.1% FA, 30-min gradient). Purification was carried out by preparative RP-HPLC (column C, 2–20% MeCN in H_2O, 0.1% FA, 30-min gradient) to give the desired FA salt (*R*)-**2** (16 mg, 24%) as a white fluffy solid. The purified product was analyzed by analytical RP-HPLC (column A, UV: λ = 214 nm) using both MeCN t_R 7.79 (5–60% MeCN in H_2O, 0.1% FA, 25-min gradient) and MeOH t_R 10.28 (0–80% MeOH in H_2O, 0.1% FA, 25 min gradient) and revealed to be of >99% purity. HRMS calcd *m/z* for $C_{33}H_{60}O_{10}N_{11}$ $[M + H]^+$ 770.4519, found 770.4521.

D-Arg-D-Tyr-(*R*)-Bgl-D-Val-D-Glu-D-Leu-D-Ala-NH_2 (*R*)-3

D-Arg-D-Tyr-(*R*)-Bgl-D-Val-D-Glu-D-Leu-D-Ala-NH_2 (*R*)-**3** was prepared on a 0.082 mmol scale, in a syringe tube following the optimized protocol as described above, using microwave-assisted annulation over 4 h, to give the desired lactam peptide TFA salt (*R*)-**3** (34 mg) in 86% crude purity as determined by analytical RP-HPLC (column B, UV: λ = 214 nm, 0–20% MeCN in H_2O, 0.1% FA, 30-min gradient). Purification was carried out by preparative RP-HPLC (column D, 0–20% MeCN in H_2O, 0.1% FA, 30-min gradient) to give the desired FA salt (*R*)-**3** (17 mg, 23%) as a white fluffy solid. The purified product was analyzed by analytical RP-HPLC (column B, UV: λ = 214 nm) using both MeCN t_R 9.55 (5–60% MeCN in H_2O, 0.1% FA, 25-min gradient) and MeOH t_R 12.99 (0–80% MeOH in H_2O, 0.1% FA, 25-min gradient) and revealed to be of >99% purity. HRMS calcd *m/z* for $C_{38}H_{62}O_{10}N_{11}$ $[M + H]^+$ 832.4676, found 832.4677.

D-Arg-D-Tyr-D-Thr-(*R*)-Bgl-D-Glu-D-Leu-D-Ala-NH_2 (*R*)-4

D-Arg-D-Tyr-D-Thr-(*R*)-Bgl-D-Glu-D-Leu-D-Ala-NH_2 (*R*)-**4** was prepared on a 0.087 mmol scale, in a syringe tube following the optimized protocol as described above, using microwave-assisted annulation over 7 h, to give the desired lactam peptide TFA salt (*R*)-**4** (62 mg) in 79% crude purity as determined by analytical RP-HPLC (column B, UV: λ = 214 nm, 0–30% MeCN in H_2O, 0.1% FA, 30-min gradient). Purification was carried out by preparative RP-HPLC (column D, 18% MeCN in H_2O, 0.1% FA, isocratic) to give the desired FA salt (*R*)-**4** (16 mg, 17%) as a white fluffy solid. The purified product was analyzed by analytical RP-HPLC (column B, UV: λ = 214 nm) using both MeCN t_R 8.00 (5–60% MeCN in H_2O, 0.1% FA, 25-min gradient) and MeOH t_R 9.93 (0–80% MeOH in H_2O, 0.1% FA, 25-min gradient) and revealed to be of >99% purity. HRMS calcd *m/z* for $C_{37}H_{60}O_{11}N_{11}$ $[M + H]^+$ 834.4468, found 834.4470.

D-Arg-D-Tyr-D-Thr-D-Val-(*R*)-Bgl-D-Leu-D-Ala-NH_2 (*R*)-5

D-Arg-D-Tyr-D-Thr-D-Val-(*R*)-Bgl-D-Leu-D-Ala-NH_2 (*R*)-**5** was prepared on a 0.098 mmol scale, in a syringe tube following the optimized protocol as described above, using microwave-assisted annulation over 4 h, to give the desired lactam peptide TFA salt (*R*)-**5** (35 mg) in 87% crude purity as determined by analytical RP-HPLC (column A, UV: λ = 214 nm, 0–80% MeCN in H_2O, 0.1% FA, 30-min gradient). Purification was carried out by preparative RP-HPLC (column C, 0–30% MeCN in H_2O, 0.1% FA, 30-min gradient) to give the desired FA salt (*R*)-**5** (6 mg, 7%) as a white fluffy solid. The purified product was analyzed by analytical RP-HPLC (column A, UV: λ = 214 nm) using both MeCN t_R 9.67 (5–60% MeCN in H_2O, 0.1% FA, 25-min gradient) and MeOH t_R 13.18 (0–80% MeOH in H_2O, 0.1% FA, 25-min gradient) and revealed to be of >99% purity. HRMS calcd *m/z* for $C_{37}H_{61}O_9N_{11}$ $[M + Na]^+$ 826.4546, found 826.4544.

D-Arg-D-Tyr-D-Thr-D-Val-D-Glu-(*R*)-Bgl-D-Ala-NH_2 (*R*)-6

D-Arg-D-Tyr-D-Thr-D-Val-D-Glu-(*R*)-Bgl-D-Ala-NH_2 (*R*)-**6** was prepared on a 0.105 mmol scale, in a syringe tube following the optimized protocol as described above, using microwave-assisted annulation over 4 h, to give the desired lactam peptide TFA salt (*R*)-**6** (35 mg) in 50% crude purity as determined by analytical RP-HPLC (column B, UV: λ = 214 nm, 0–60% MeCN in H_2O, 0.1% FA, 25-min gradient). Purification was carried out by preparative RP-HPLC (column D, 0–20% MeCN in H_2O, 0.1% FA, 30-min gradient) to give the desired FA salt (*R*)-**6** (13 mg, 14%) as a white fluffy solid. The purified product was analyzed by analytical RP-HPLC (column B, UV: λ = 214 nm) using both MeCN t_R 7.27 (5–60% MeCN in H_2O, 0.1% FA, 25-min gradient) and MeOH t_R 9.04 (0–80% MeOH in H_2O, 0.1% FA, 25-min gradient) and revealed to be of >99% purity. HRMS calcd *m/z* for $C_{36}H_{58}O_{11}N_{11}$ $[M + H]^+$ 820.4312, found 820.4313.

(*S*)-Bgl-D-Tyr-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2 (*S*)-1

(*S*)-Bgl-D-Tyr-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2 (*S*)-**1** was prepared on a 0.102 mmol scale, in a syringe tube following the optimized protocol as described above, using microwave-assisted annulation over 10 h, to give the desired lactam peptide TFA salt (*S*)-**1** (34 mg) in 70% crude purity as determined by analytical RP-HPLC (column A, UV: $\lambda = 214$ nm, 0–80% MeCN in H_2O, 0.1% FA, 30-min gradient). Purification was carried out by preparative RP-HPLC (column C, 10–30% MeCN in H_2O, 0.1% FA, 30-min gradient) to give the desired FA salt (*S*)-**1** (11 mg, 13%) as a white fluffy solid. The purified product was analyzed by analytical RP-HPLC (column A, UV: $\lambda = 214$ nm) using both MeCN t_R 12.00 (5–60% MeCN in H_2O, 0.1% FA, 25-min gradient) and MeOH t_R 19.49 (0–60% MeOH in H_2O, 0.1% FA, 25-min gradient) and revealed to be of >99% purity. HRMS calcd *m/z* for $C_{36}H_{57}O_{11}N_8$ $[M + H]^+$ 777.4141, found 777.4138.

D-Arg-(*S*)-Bgl-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2 (*S*)-2

D-Arg-(*S*)-Bgl-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2 (*S*)-**2** was prepared on a 0.105 mmol scale, in a syringe tube following the optimized protocol as described above, using microwave-assisted annulation over 10 h, to give the desired lactam peptide TFA salt (*S*)-**2** (33 mg) in 82% crude purity as determined by analytical RP-HPLC (column A, UV: $\lambda = 214$ nm, 0–20% MeCN in H_2O, 0.1% FA, 30 min gradient). Purification was carried out by preparative RP-HPLC (column C, 0–20% MeCN in H_2O, 0.1% FA, 30-min gradient) to give the desired FA salt (*S*)-**2** (13 mg, 16%) as a white fluffy solid. The purified product was analyzed by analytical RP-HPLC (column A, UV: $\lambda = 214$ nm) using both MeCN t_R 7.14 (5–60% MeCN in H_2O, 0.1% FA, 25-min gradient) and MeOH t_R 8.62 (0–80% MeOH in H_2O, 0.1% FA, 25-min gradient) and revealed to be of >99% purity. HRMS calcd *m/z* for $C_{33}H_{60}O_{10}N_{11}$ $[M + H]^+$ 770.4519, found 770.4518.

D-Arg-D-Tyr-(*S*)-Bgl-D-Val-D-Glu-D-Leu-D-Ala-NH_2 (*S*)-3

D-Arg-D-Tyr-(*S*)-Bgl-D-Val-D-Glu-D-Leu-D-Ala-NH_2 (*S*)-**3** was prepared on a 0.105 mmol scale, in a syringe tube following the optimized protocol as described above, using microwave-assisted annulation over 8 h, to give the desired lactam peptide TFA salt (*S*)-**3** (52 mg) in 60% crude purity as determined by analytical RP-HPLC (column A, UV: $\lambda = 214$ nm, 0–80% MeCN in H_2O, 0.1% FA, 30-min gradient). Purification was carried out by preparative RP-HPLC (column C, 5–15% MeCN in H_2O, 0.1% FA, 50 min gradient) to give the desired FA salt (*S*)-**3** (15 mg, 17%) as a white fluffy solid. The purified product was analyzed by analytical RP-HPLC (column A, UV: $\lambda = 214$ nm) using both MeCN t_R 9.43 (5–60% MeCN in H_2O, 0.1% FA, 25-min gradient) and MeOH t_R 14.71 (0–60% MeOH in H_2O, 0.1% FA, 25-min gradient) and revealed to be of >99% purity. HRMS calcd *m/z* for $C_{38}H_{62}O_{10}N_{11}$ $[M + H]^+$ 832.4676, found 832.4674.

D-Arg-D-Tyr-D-Thr-(*S*)-Bgl-D-Glu-D-Leu-D-Ala-NH_2 (*S*)-4

D-Arg-D-Tyr-D-Thr-(*S*)-Bgl-D-Glu-D-Leu-D-Ala-NH_2 (*S*)-**4** was prepared on a 0.105 mmol scale, in a syringe tube following the optimized protocol as described above, using microwave-assisted annulation over 7 h, to give the desired lactam peptide TFA salt (*S*)-**4** (28 mg) in 87% crude purity as determined by analytical RP-HPLC (column A, UV: $\lambda = 214$ nm, 0–80% MeCN in H_2O, 0.1% FA, 30-min gradient). Purification was carried out by preparative RP-HPLC (column C, 0–20% MeCN in H_2O, 0.1% FA, 20-min gradient) to give the desired FA salt (*S*)-**4** (12 mg, 14%) as a white fluffy solid. The purified product was analyzed by analytical RP-HPLC (column A, UV: $\lambda = 214$ nm) using both MeCN t_R 7.56 (5–60% MeCN in H_2O, 0.1% FA, 25-min gradient) and MeOH t_R 8.91 (0–80% MeOH in H_2O, 0.1% FA, 25-min gradient) and revealed to be of >99% purity. HRMS calcd *m/z* for $C_{37}H_{60}O_{11}N_{11}$ $[M + H]^+$ 834.4468, found 834.4469.

D-Arg-D-Tyr-D-Thr-D-Val-(*S*)-Bgl-D-Leu-D-Ala-NH_2 (*S*)-5

D-Arg-D-Tyr-D-Thr-D-Val-(*S*)-Bgl-D-Leu-D-Ala-NH_2 (*S*)-**5** was prepared on a 0.105 mmol scale, in a syringe tube following the optimized protocol as described above, using microwave-assisted annulation over 4 h, to give the desired lactam peptide TFA salt (*S*)-**5** (40 mg) in 90% crude purity as determined by analytical RP-HPLC (column A, UV: $\lambda = 214$ nm, 0–80% MeCN in H_2O, 0.1% FA, 30-min gradient). Purification was carried out by preparative RP-HPLC (column C, 0–20% MeCN in H_2O, 0.1% FA, 30-min gradient) to give the desired FA salt (*S*)-**5** (11 mg, 13%) as a white fluffy solid. The purified product was analyzed by analytical RP-HPLC (column A, UV: $\lambda = 214$ nm) using both MeCN t_R 9.48 (5–60% MeCN in H_2O, 0.1% FA, 25-min gradient) and MeOH t_R 10.47 (0–80% MeOH in H_2O, 0.1% FA, 25-min gradient) and revealed to be of >99% purity. HRMS calcd *m/z* for $C_{37}H_{61}O_9N_{11}$ $[M + H]^+$ 804.4726, found 804.4724.

D-Arg-D-Tyr-D-Thr-D-Val-D-Glu-(*S*)-Bgl-D-Ala-NH_2 (*S*)-6

D-Arg-D-Tyr-D-Thr-D-Val-D-Glu-(*S*)-Bgl-D-Ala-NH_2 (*S*)-**6** was prepared on a 0.105 mmol scale, in a syringe tube following the optimized protocol as described above, using microwave-assisted annulation over 4 h, to give the desired lactam peptide TFA salt (*S*)-**6** (29 mg) in 96% crude purity as determined by analytical RP-HPLC (column A, UV: $\lambda = 214$ nm, 0–20% MeCN in H_2O, 0.1% FA, 30-min gradient). Purification was carried out by preparative RP-HPLC (column C, 0–20% MeCN in H_2O, 0.1% FA, 30-min gradient) to give the desired FA salt (*S*)-**6** (10 mg, 11%) as a white fluffy solid. The purified product was analyzed by analytical RP-HPLC (column A, UV: $\lambda = 214$ nm) using both MeCN t_R 7.6 (5–60% MeCN in H_2O, 0.1% FA, 25-min gradient) and MeOH t_R 8.9 (0–80% MeOH in H_2O, 0.1% FA, 25-min gradient) and revealed to be of >99% purity. HRMS calcd *m/z* for $C_{36}H_{58}O_{11}N_{11}$ $[M + H]^+$ 820.4312, found 820.4318.

Immune Cell Proliferation Assay

Immune cell proliferation was assessed by DNA (nuclear and mitochondrial) intercalation of CyQUANT NF dye (Invitrogen, Carlsbad, CA, USA) [29]. Essentially, human TF-1 cells (CRL-2003, American Type Culture Collection) were cultured in complete RPMI 1640 (WISENT Inc., Montreal, Quebec, Canada) supplemented with GM-CSF (2 ng/ml, PeptroTech, Rocky Hill, NJ, USA). Cells were reincorporated into RPMI 1640 medium deprived of phenol red (Gibco Invitrogen) and of growth factors [i.e. fetal bovine serum (FBS) and GM-CSF] for 18 h and split (5×10^3 cells/well) into 96-well non-adherent flat bottom culture plates (Sarstedt, Nümbrecht, Germany). Following 15 min preincubation with peptide or peptidomimetic (1 µM), the cells were treated with human IL-1β (25 ng/ml, PeproTech) and reincubated. After 48 h, the cells were centrifuged for 7 min at 300g, reincorporated into Hank's Buffered Salt Solution (1X HBSS, 50 µL/well, Gibco Invitrogen) and transferred into a 96-well polystyrene black plate (Corning Incorporated, Corning, NY, USA). An additional 50 µl

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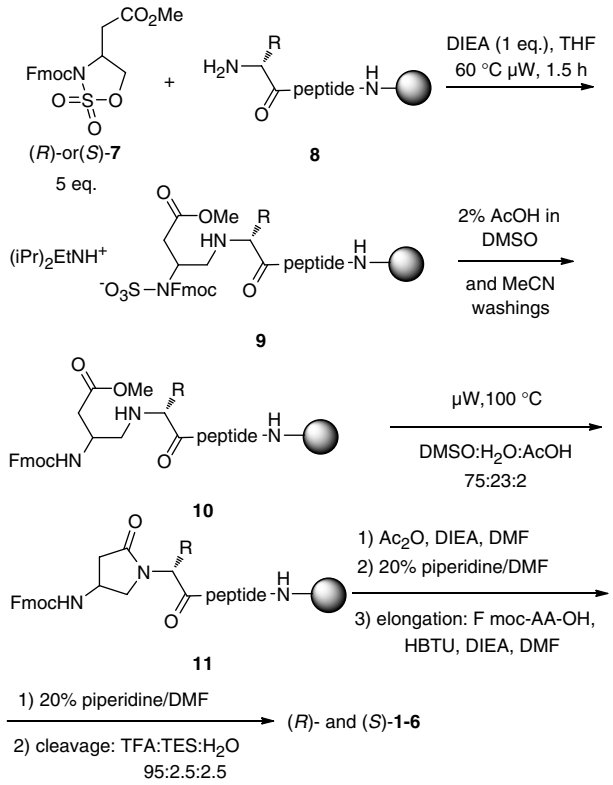

Scheme 1. Optimized protocol for Bgl installment.

solution made of CyQUANT NF dye (component A) and CyQUANT NF delivery agent (component B) in HBSS was added to the cells in each well, which were covered with aluminum foil and incubated for 45 min. Fluorescence intensity was measured using a microplate reader-fluorimeter (Perkin Elmer Wallac Envision, Waltham, MA, USA, FITC 485 and FITC 531 filters) with excitation at 485 nm and emission detection at 530 nm. Experiments were repeated in triplicate and results were analyzed by one-way analysis of variance (ANOVA) factoring for treatments, and data are presented as mean ± standard error of the mean (SEM).

CD Analyses

Far-UV CD spectra of 101.10 and analogs were all recorded on a Chirascan CD Spectrometer (Applied Photophysics Ltd., Surrey, UK) using a 1.0-cm pathlength quartz cuvette containing 20 μM of peptide dissolved in Chromspec filtered Milli-Q water. Instrumental settings were fixed as bandwidth of 1 nm, step size of 0.5 nm, sampling time of 2 s.

Results and Discussion

Chemistry

In studying the synthesis of Bgl analogs of GHRP-6 [21], the *N*-alkylation/lactam annulation process was optimized using five-membered cyclic sulfamidates (*R*)- and (*S*)-**7** (Scheme 1), which were prepared from D- and L-aspartic acid, respectively. In particular, the use of DIEA increased conversion during the alkylation of the resin-bound peptide with sulfamidate **7** and employment of a DMSO : H_2O : AcOH solvent mixture-enhanced lactam formation during the microwave-assisted ring closure [27].

The Bgl analogs of 101.10 were synthesized on Rink amide MBHA resin in syringe tubes. Initially, standard protocols for parallel solid-phase peptide synthesis using Fmoc protection were employed to synthesize the peptide chain [30,31]. The (*S*)- and (*R*)-Bgl residues were then installed using the optimized protocol described in the experimental section (Scheme 1) [27]. In brief, after Fmoc removal, the resin was transferred into a 2-ml glass microwave vessel, treated with a solution of cyclic sulfamidate **7** (5 equiv.) and DIEA (1 equiv.) in THF (2 ml) and irradiated in a sealed reactor with microwave heating at 60 °C for 1.5 h. To decompose the resulting sulfamate salt **9**, the resin was treated with 2% AcOH in DMSO, before irradiation with microwave heating at 100 °C in a DMSO : H_2O : AcOH (75 : 23 : 2, 2 ml) mixture for a minimum time of 4 h (Table 1). After capping with acetic anhydride and DIEA in DMF, peptide synthesis was completed using standard Fmoc chemistry protocols. Bgl peptides (*R*)- and (*S*)-**1**–**6** were cleaved off the resin using a TFA : H_2O : TES (95 : 2.5 : 2.5, v/v/v) mixture, precipitated in cold diethyl ether and purified by preparative reverse phase high performance liquid chromatography (RP-HPLC) to give white fluffy solids (Table 1).

Proximity to the solid support appeared to be an important parameter for success of the alkylation reaction with sulfamidate **7**, and better yields were obtained in alkylations further from the polymer matrix. Product corresponding to bis-alkylation accounted for <10% of the total UV signal at 214 nm, as measured by RP-HPLC analysis of cleaved samples. Acetyl capping after lactam formation improved the purification of crude products during precipitation in cold diethyl ether by enhancing the solubility of unsuccessful alkylation products. Microwave-assisted lactam formation required longer reaction times (>4 h) in the syntheses of (*R*)-**2** and (*R*)-**4**, as well as (*S*)-**1**–**4**, presumably due to steric effects of bulky β-alkyl branched and protected side chains. Moreover, the configuration of sulfamidate **7** influenced the rate of lactam formation. On the D-Tyr(OtBu) residue, 10 and 4 h of microwave heating were required, respectively, to achieve 90% conversion in the cases of (*R*)- and (*S*)-**1**. On the other hand, lactam formation proceeded faster with the (*R*)-isomer on D-Thr(OtBu), taking 6 h, instead of 10 h in the synthesis of (*S*)- **2**.

Biology

The efficacy of 101.10 lactam analogs was ascertained by measuring their influence on IL-1β-induced human thymocyte (TF-1 cell line) proliferation [21,22]. Although this was previously assessed by the incorporation of tritiated thymidine [19], we now present a novel fluorescence detection method, which provides comparable results, but relies on the enhancement of fluorescence upon intercalation of a cyanine dye (CyQUANT NF™) into DNA of cultured cells. More cost effective without the need for disposal of radionucleotide waste, the fluorescence-based method is better suited for high throughput screening in microplate assays, because low quantum-yield reporter molecules are eliminated. Experimental sensitivity and reproducibility were thus enhanced for studying structure-activity relationships.

101.10 and Bgl-peptides (*R*)- and (*S*)-**1**–**6** were screened for efficacy in inhibiting TF-1 proliferation induced by addition of IL-1β, which was reported as an average percentage of the control proliferation in Figure 3. The normal proliferation of TF-1 cells due to the endogenous secretion of growth factors such as IL-1β was set as control. Cells in the IL-1β wells exhibited

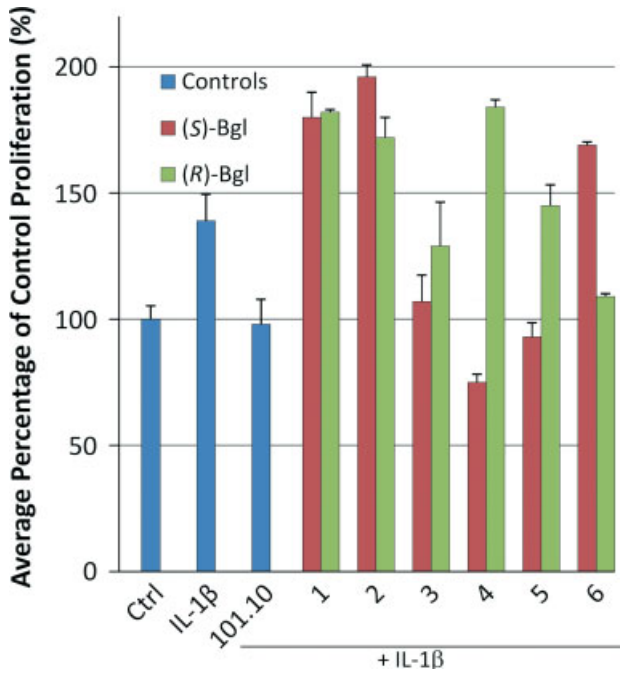

Figure 3. Inhibition of IL-1 induced thymocyte cell proliferation by (*R*)- and (*S*)-**1**–**6**.

an increased proliferation rate because of the added amount of exogenous IL-1β. In general, the (*R*)-Bgl analogs exhibited lower inhibitory activity compared with their (*S*)-Bgl counterparts. Replacement at the *N*-terminal residues D-Arg^1 and D-Tyr^2 with Bgl of both configurations diminished profoundly efficacy (1.7- to 2-fold), indicating that the side chains and orientation of the terminal amine may be important for activity. Relative to 101.10, replacement of D-Thr^3 and D-Glu^5 with (*S*)-Bgl, as well as D-Leu^6 by (*R*)-Bgl afforded, respectively, analogs (*S*)-**3**, (*S*)-**5** and (*R*)-**6**, which all had similar efficacy as the parent peptide. Substitution of D-Val^4 by (*S*)-Bgl produced (*S*)-**4**, which exhibited a statistically relevant increase in efficacy compared with 101.10 by inhibiting TF-1 proliferation under the control level (75%), indicating that (*S*)-**4** blocked the proliferative effects of both exogenous and endogenous IL-1β.

The influence of the stereochemistry of the Bgl residue had varying effects on the activity of the peptide dependent on its place in the sequence. Swapping (*S*)-Bgl for (*R*)-Bgl shows little impact on the first three positions, whereas the effect is more drastic on positions 4–6. Centered on position 3, 4 and 5, the (*S*)-Bgl analogs show an inverse bell-shaped curve with a minimum for position 4. The curve shape is reversed on these positions for the (*R*)-Bgl analogs, with a maximum on position 4. These results stress the fact that the correct orientation of folding in the center part of the peptide may be a determinant for activity. Previously, substitution of Val^4 for the α-amino lactam (*R*)-Agl in 101.10 gave a similar beneficial effect on activity as that displayed by the (*S*)-Bgl^4 counterpart (*S*)-**4**. Moreover, the analogous (*S*)-Agl^4 analog exhibited a loss of activity similar to the (*R*)-Bgl^4 derivative (*R*)-**4** [21,22]. This is concordant with earlier studies using both types of amino lactams [32], and may be rationalized by receptor tolerance of a subtle change in ligand geometry created by moving the amine on the same face of the ring from the α- to β-position without changing the orientation of the lactam carbonyl (Figure 1).

Conformational Analysis by CD Spectroscopy

The conformational effects of the Bgl moiety on the peptide were assessed by CD spectroscopy in water. Note that relative to their enantiomeric counterparts, D-peptides such as 101.10 and its lactam analogs exhibited curve shapes inverted from those of L-peptides, indicative of mirror image secondary structures [33]. Random coil CD curves featuring a positive maximum around 198 nm indicated disordered structures for the parent peptide and (*R*)-**6**, as well as (*R*)-**4** (Figure 4). Bgl peptides (*S*)-**4** and (*R*)-**5**, which had CD curves featuring a positive maximum around 198 nm and negative maximum at 215 nm in their spectra, are suspected to adopt discrete PPII helical conformations. On the other hand, the CD signature of (*S*)-**3** displayed distinct positive maximum at 198 and 228 nm and negative maximum at 208 nm, suggestive of a β-turn conformation. A similarly shaped CD curve was observed for (*R*)-**3** but did not cross the axis, consequently bringing the ellipticity into question. The CD spectrum of (*S*)-**6** displayed an α-helical signature, exhibiting two negative maxima at 195 and 210 nm and a positive maximum at 220 nm [34]. Similarly, the presence of weaker negative and positive bands around 210 and 220 nm in the spectrum of analog (*S*)-**5** may indicate a discrete amount of α-helical conformation.

Relating activity to curve shape and conformation, one may conclude that substitution of D-Thr^3 by (*S*)-Bgl confers turn structure favorable for biological activity, in spite of the loss of the hydroxyethyl side chain. Similarly, replacement of D-Val^4 by (*S*)-Bgl [(*S*)-**4**] induces PPII helicity which may enhance the activity. In contrast, the less active (*R*)-Bgl^4 [(*R*)-**4**] analog exhibited a CD curve characteristic of a random coil. Finally, introduction of (*S*)-Bgl at the *C*-terminal of 101.10 may force the peptide backbone to adopt an α-helical conformation detrimental to the biological activity.

Conclusion

Twelve Bgl analogs of the D-heptapeptide 101.10 [(*R*)- and (*S*)-**1**–**6**] were synthesized, with high crude purity and purified to >99% in good overall yields, using an optimized solid-phase methodology featuring *N*-alkylation and lactam annulation with cyclic sulfamidate **7**. Evaluation of (*R*)- and (*S*)-**1**–**6** for inhibitory activity to IL-1β-induced TF-1 cell proliferation and CD spectroscopy of certain analogs has provided information concerning the requirements for the biological activity of the parent peptide. Considering a balance between the loss of side chain and the conformational effects induced by the Bgl residues, the increased activity of [(*S*)-Bgl^4] 101.10 [(*S*)-**4**] and its PPII type CD curve shape, as well as the retained potency of [(*S*)-Bgl^3] 101.10 [(*S*)-**3**] and its β-turn curve shape indicate that the presence of a fold about the central residues of the parent peptide may be important for activity. Lactam scanning has thus proven useful for enhancing peptide potency and identifying the conformational requirements for biological activity.

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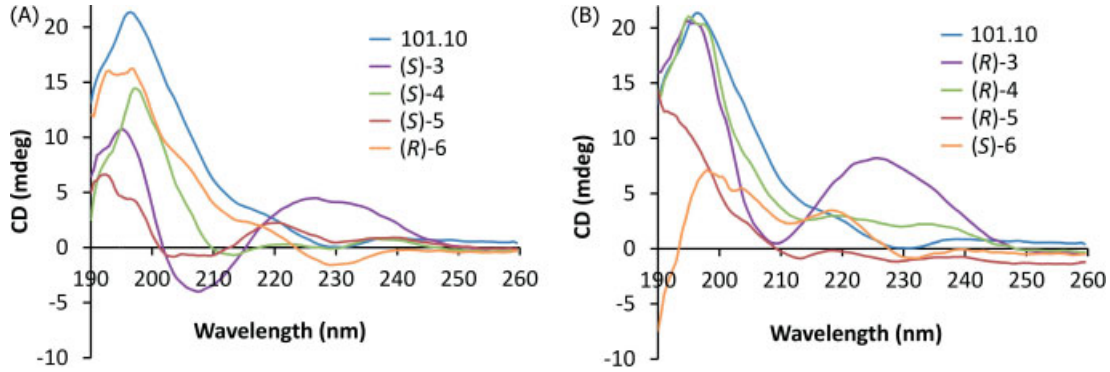

Figure 4. CD of 101.10, active analogs (*S*)-**3**–**5** and (*R*)-**6** (**A**), and inactive analogs (*R*)-**3**–**5** and (*S*)-**6** (**B**).

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