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PEPTIDE INHIBITORS OF IKB PROTEASE: MODIFICATION OF THE C-TERMINI OF Z-LLF-CHO

Mark J. Suto,* Robert W. Sullivan, and Lynn J. Ransone
Signal Pharmaceuticals, Inc., 5555 Oberlin Drive
San Diego, California 92121

Abstract. A series of tripeptides (Z-LLF-R) with various modifications at their C-terminus were synthesized and evaluated for their ability to prevent the activation of NF-κB through inhibition of IκB protease. Of the compounds evaluated only the C-terminal aldehydes **5a,b** were active in our Jurkat T-cell based assay. Compound **5a** also decreased IL-2 and IL-8 levels in these cells indicating that inhibitors of IκB protease can have an effect on various signaling pathways.

Introduction

NF-κB is an inducible transcription factor that regulates a number of proinflammatory proteins and cytokines.[1] A recent review demonstrates the critical role of NF-κB in a number of pathological disorders and therefore its potential as a target for regulating inflammatory processes.[2] NF-κB is normally found in the cytosol as a heterodimer complexed to IκB and cannot translocate into the nucleus in this form. Recent studies have shown that a variety of steps including phosphorylation, ubiquination, and degradation of IκB must occur before NF-κB is released and able to translocate to the nucleus.[3] The regulation of this process can occur at many levels involving a variety of inducers and signaling pathways.

One approach to the regulation of NF-κB activation would be to block the proteolytic degradation of IκB.[4] The protease responsible for this degradation (IκB protease) is found as part of a complex proteasome that is responsible for the degradation of a number of proteins, one of which is IκB.[5] The proteasome has many distinct catalytic functions resembling several types of known proteases. Recent studies have shown that small peptides can reversibly inhibit the degradation a number of these proteases. In particular, the tripeptide CBZ-Leu-Leu-Phe-CHO (Z-LLF-CHO, **5**) was shown to prevent the degradation of phosphorylated IκB[6] and thus provides a potential approach to the regulation of a number of NF-κB mediated signaling events.

In an effort to more fully understand the role of IκB protease, its potential use as a drug discovery target, and the specificity of the proteasome in general, we synthesized a series of C-terminal-modified tripeptides related to Z-LLF-CHO, and evaluated them for their ability to block NF-κB activation.

Chemistry

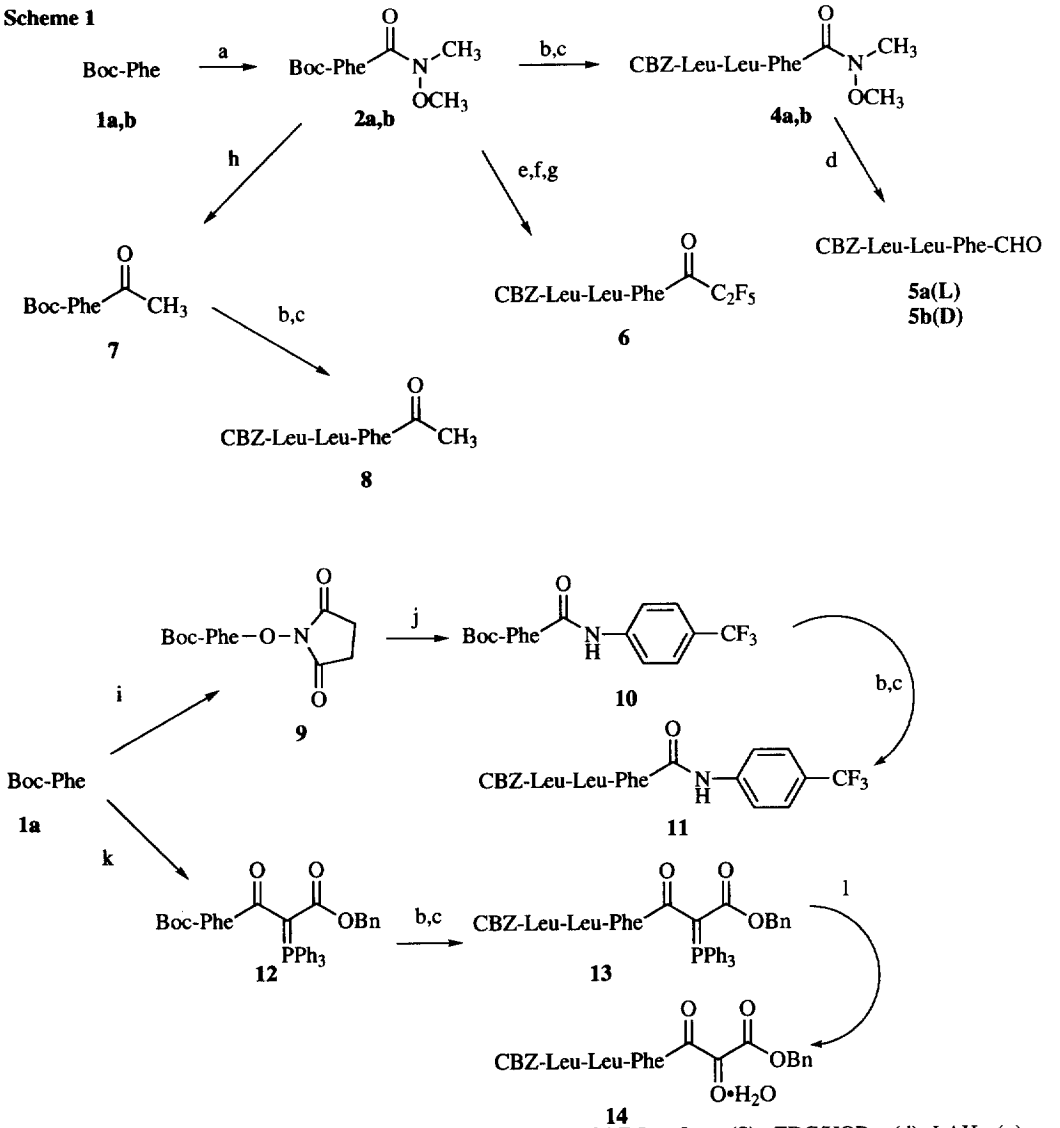
The compounds synthesized[14] are listed in Table 1 and were selected based upon published data on various inhibitors of proteases. The goal was to determine if functionalities other than the aldehyde could selectively inhibit IκB protease and thus prevent the activation of NF-κB. The rationale for examining other C-terminal functionalities is due to the fact that peptide aldehydes in general suffer from a number of deficiencies including a lack of stability, ease of epimerization, and difficulty in preparation. Structurally related aldehydes have also been shown to have inhibitory activity in several biological systems, including several related proteases.[13]

In an effort to address these questions, we first prepared enantiomerically pure samples of both the L and D isomers of Z-LLF-CHO. The synthesis starts with each enantiomer (R and S) of Boc-phenylalanine **1 a** and **b**[7] (Scheme 1). They were converted to the corresponding N,O-dimethylhydroxy amide (**2 a** and **b**), using standard techniques.[8] The Boc-groups were removed and the amino acids were coupled to the commercially- available dipeptide CBZ-lecine-leucine[9] **3** to provide the tripeptides **4a** and **b**. Reduction of both **4a** and **b** with LAH provided each of the desired aldehydes **5a** and **b**, which were clearly distinguishable by both ^{1}H NMR and HPLC.

The pentafluoroethyl ketone **6** was obtained by treatment of **2a** with pentafluoroethyl iodide/CH_3Li[10] and coupling to **3** after removal of the Boc group. The synthesis of the methylketone **8** proceeded via a similar route starting with Weinreb's amide **2** (Scheme 1). Treatment of **2** with MeMgBr in THF provided the methyl ketone **7** in 58% yield. Deprotection and coupling to **3** provided the desired tripeptide **8**.

The 4-CF_3 phenyl amide **11** was also prepared. As shown in Scheme 1, the N-hydroxysuccinamide of Boc-phenylalanine **9** was synthesized, and then reacted with p-trifluoromethyl aniline to provide the amide **10**. The Boc-group was removed and the amino acid coupled with **3** to provide **11**. A similar procedure was used to prepare the dipeptide amide **18** (Scheme 2). Boc-Leucine **15** was reacted with N-hydroxysuccinamide to give the activated amino acid **16**. Treatment of **16** with 3′-aminoacetophenone provided **17** in poor yield, however enough was obtained to couple to CBZ-leucine to give **18**. N-Hydroxysuccinamide was used as an activating group because more standard procedures (CDI, EDC, acid chloride) failed to provide sufficient quantities of the desired product.

Scheme 1



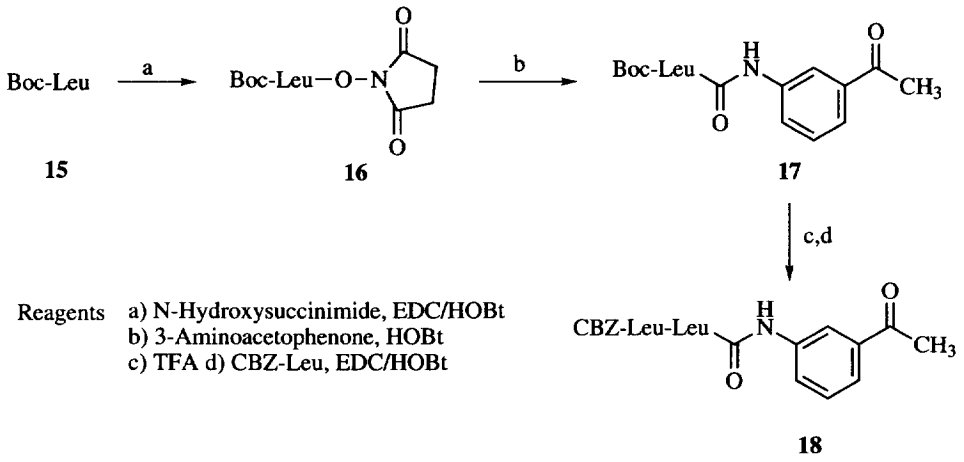
Reagents: (a) EDC/HOBt, $HN(OCH_3)(CH_3)$; (b) TFA; (c) CBZ-Leu-Leu (**3**), EDC/HOBt; (d) LAH; (e) $C_2F_5I/CH_3Li{\cdot}LiBr$; (f) HCl; (g) CBZ-Leu-Leu (**3**), isobutyl chloroformate; (h) CH_3MgBr; (i) N-Hydroxysuccinimide, EDC/HOBt; (j) 4-Trifluoromethylaniline, HOBt; (k) $Ph_3P = CHCO_2Bn$; (l) Oxone

One last compound that was synthesized was the tricarbonyl derivative **14** (Scheme 1). The tricarbonyl is believed to have the ability to act similarly to the aldehyde in that it can form a hemiacetal type intermediate with hydroxy groups.[11] The synthesis of **14** also started with Boc-phenylalanine **1a**. Other approaches, such as the synthesis of the phosphine tripeptide directly were not successful. The phosphine derivative **12** was

formed in a 60% yield using the procedure of Wasserman.[11] Deprotection and coupling to **3** gave the tripeptide **13**, which was oxidized with Oxone™. This reaction was somewhat problematic in that it was very sluggish and required a long reaction time. However, a small amount of the desired product **14** was obtained after chromatography.

In addition to the desired compounds, we prepared benzyloxycarbonyl-(2-naththyl)alanine-(1-naphthyl)alanine-leucinal **19** starting from Boc-leucine.[12] This compound was reported to be a potent inhibitor of the proteasome and therefore we wanted to compare it to compound **5a**.

Scheme 2



Biological Evaluation

All of the compounds in Table 1 were evaluated in our cell-based assay of NF-κB activation. Human Jurkat T-cells stably expressing an NF-κB binding site fused to a minimal promoter driving luciferase were used. Cells were split to 3×10^5 cells/mL every 2-3 days. The cells were counted, resuspended in fresh medium and plated in 96-well plates 18 h prior to starting the experiment. Compounds listed in Table 1 were dissolved at various concentrations (DMSO) and added to the cell plates. After a 30 min preincubation, 50 ng/mL of PMA and 1 mg/mL of PHA was added to each well, and the cells were incubated for an additional 5 h at 37 °C. Cells were harvested by centrifugation, 60 μL of lysis buffer was added to each well followed by a 0.25 h incubation. Forty microliters of each cell extract was transferred to a black 96-well plate and 50 μL of luciferase substrate buffer was added. Luminescence was measured using a Packard TopCount. The data is expressed as IC_{50} values (Table 1) and is the average of at least two determinations.

In addition, compound **5a** was evaluated for its effects on cytokine production in the Jurkat cells. Supernatants from the above experiments were saved, and IL-2 and IL-8 levels were determined by ELISA (ENDOGEN). The IC_{50} value for IL-2 is 0.9 μM and for IL-8, 2 μM.

Table 1

Compound	NFκB (IC_{50}, μM)	Compound	NFκB (IC_{50}, μM)
4a (L)	18	8	>30
4b (D)	>30	11	>30
5a (L)	2	14	20
5b (D)	2	18	>10
6	>30	19	3

Results

As shown in Table 1, the only compounds having any effect on the activation of NF-κB were the aldehydes **5a**, **5b**, and **19**. Both the L and D isomers were equally active. Although other C-terminal modifications such as the trifluoromethylketones and p-trifluoromethylaniline derivatives have been used as elastase inhibitors[13] and the tricarbonyl and methyl ketones have been used as chymotrypsin inhibitors, none were able to prevent activation of NF-κB at the doses tested. Compounds such as **2a** and **2b**, which in some ways mimic the proposed transition state of aldehyde-type inhibitors, were also inactive as is the corresponding alcohol. This suggests that IκB protease is sensitive only to peptide aldehydes. Since peptide aldehydes can affect many types of proteases, it may be difficult to identify compounds selective for IκB protease. In addition, evaluation of cytokine levels from the supernatants indicate that compound **5a** also affects IL-2 in addition to the expected effects on IL-8. This indicates that the peptide aldehydes are capable of inhibiting other cellular functions besides NF-κB. Recently, a large series of peptide aldehydes was prepared and evaluated as inhibitors of the 20S proteasome.[12] One of the more potent compounds reported was benzyloxycarbonyl-(2-naphthyl)alanine-(1-naphthyl)alanine-leucinal **19**. This compound also had activity in our cell-based assays at comparable doses to compounds **5a** and **b**, although it was reported to be a much more potent inhibitor of the 20S proteasome.[12]

In summary, we have shown that peptide aldehydes are capable of inhibiting the activation of NF-κB, presumably through inhibition of IκB protease. These compounds inhibit IL-8 levels as expected, but they also inhibit IL-2 levels, indicating an effect on other cellular processes besides NF-κB. Further studies have shown that compounds **5a** and **19** also inhibit the activation of AP-1, which may account for the activity seen against IL-2.[6] This data indicates that the effect seen by the tripeptide aldehydes on NF-κB may occur through other signaling pathways in addition to IκB protease. Currently, we are examining these compounds further to better understand their effects on multiple signaling pathways and as tools for the study of these pathways in general. Efforts are also continuing towards identifying compounds capable of selectively modulating NF-κB activity.

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14. All compounds had ^{1}H NMR consistent with the proposed structures and HPLC (>95% purity, Microsorb-MV RF C18 5 mm column, 2.0 mL/min flow, 65/35/0.1 CH_3CN/H_2O/TFA. Compound **4a**; mp 130-131 °C; FABMS *m/e* 569.4; Compound **4b**; FABMS *m/e* 569.4; Compound **5a**; mp 58-59 °C; FABMS *m/e* 510.3; Compound **5b**; FABMS *m/e* 510.3; Compound **6**; mp 135-137 °C; FABMS 628.3; Compound **8**; FABMS *m/e* 546.3; Compound **11**; mp 237-238 °C; FABMS *m/e* 669.2; Compound **14**; FABMS *m/e* 672.4; Compound **18**; FABMS *m/e* 496.2; Compound **19**; FABMS *m/e* 643.5.

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