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Identification of TNF-α Inhibitors from a Split-Pool Library Based on a Tyrosine-Proline Peptidomimetic Scaffold

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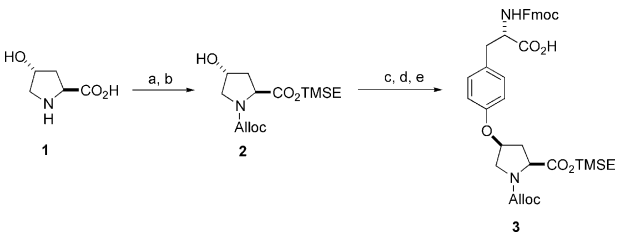
Abstract—The design and synthesis of a combinatorial library based on a 4-aryloxyproline scaffold with tyrosine as the aryl portion is described. The 1728 member library was prepared using the split-pool method to generate pools of compounds. Screening of the library components as mixtures followed by deconvolution led to the discovery of novel inhibitors of TNF-α induced apoptosis.

The design of combinatorial libraries for use as screening tools is now a firmly entrenched method in the field of medicinal chemistry.[1] Over the past decade or so many strategies have been pursued that combine the design and synthesis of combinatorial libraries with biological screening efforts. One of the earliest approaches consisted of performing a split-pool synthesis of a library and assaying the subsequent mixtures for biological activity.[2] This proved to be a very valuable method, however it can be hampered by high levels of false positives due in large part to additive effects arising from the screening of mixtures. Nonetheless, a number of viable medicinal chemistry hits have been discovered using this method.[1,2] As part of our program to generate a diverse collection of combinatorial libraries for screening, we designed an aryloxy proline scaffold based on a tyrosine-proline (Tyr-Pro) peptidomimetic onto which we could easily append a range of diversity elements. Substituted 4-aryloxy prolines have been reported, but library scaffolds based on tyrosine as the aryl portion are novel.[3,4] We report herein the results of our efforts including the synthesis of the scaffold, the generation of a 1728 member split-pool library and the identification of discrete compounds possessing anti-TNF-α activity.

The synthesis began with protection of L-*trans*-4-hydroxyproline **1** as the Alloc carbamate using allyl chloroformate under Schotten–Baumen conditions (Scheme 1).[5] The acid was then esterified using trimethylsilylethanol and EDCI/HOBT/DIEA. After chromatography the TMSE ester was obtained in 53% yield from hydroxyproline. The next step is a Mitsunobu coupling utilizing a protected form of tyrosine to generate the aryl ether.[6] Initial efforts focused on utilizing the *tert*-butyl ester of Fmoc-Tyr but these reactions invariably resulted in the isolation of a mixture of the deprotected and Fmoc protected aryloxyprolines. It is known that Mitsunobu conditions are sufficiently basic for removal of Fmoc protecting groups,[7] and our hopes of overcoming the low yields due to these side reactions were not realized. We next examined whether Tyr(*t*Bu), which is unprotected at the alpha amine, would suffice as a substrate for the reaction. We were pleased to see that addition of Tyr(*t*Bu) to a THF solution of **2** in the presence of triphenylphosphine and DEAD followed by refluxing for 30 min provided the coupled product in acceptable yield. Purification of the product proved to be difficult and flash chromatography afforded a mix-



Scheme 1. Reagents and conditions: (a) Alloc-Cl, Na_2CO_3, dioxane/H_2O; (b) TMSE-OH, EDCI, HOBT, DIEA, 53% from **1**; (c) PPh_3, DEAD, Tyr(*t*Bu), THF, 60 °C, 30 min; (d) Fmoc-Cl, NMM, CH_2Cl_2; (e) SiO_2, toluene, 110 °C, 26% from **2**.

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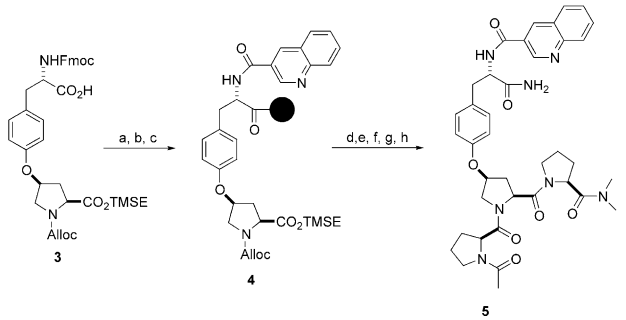
ture of the aryloxyproline and Tyr(*t*Bu) accompanied by a small amount of dicarbethoxy hydrazine. This mixture was then treated with Fmoc-Cl in the presence of NMM followed by flash chromatography to afford a mixture of the fully protected scaffold and Fmoc-Tyr(*t*Bu). Selective deprotection of the *tert*-butyl ester was accomplished using a novel cleavage strategy developed in these labs.[8] Thus, treating a toluene solution of the mixture with silica gel at reflux for 1.5 h resulted in clean conversion to carboxylic acid **3**. At this stage removal of residual Fmoc-Tyr was uneventful via flash chromatography, and the scaffold **3** was isolated in 26% overall yield from **2**.

With the scaffold in hand, we were poised to consider the chemistry and the diversity elements that we required for the synthesis of the library. Amide bond formation is extensively utilized in combinatorial chemistry due both to the hardiness of the chemistry and the vast diversity attainable from carboxylic acids and amines, and we opted to pursue this chemistry to prepare the library. The proposed chemistry was prototyped on solid phase in order to verify the robustness of the chemistry and to identify any synthetic challenges that may arise during library synthesis.

Solid phase prototyping began with attachment of the scaffold (3 equiv based on resin loading) onto Tentagel SRAM NH_2 resin using a standard coupling cocktail of 3 equiv HATU[9] and 7.5 equiv NMM in DMF (Scheme 2). Each step in the solid phase synthesis was performed twice to ensure complete reaction. After attachment onto the resin, the tyrosine amino group was deprotected and the liberated amine was coupled to 3-quinoline carboxylic acid using the standard coupling conditions to afford amide **4**. Removal of the trimethylsilylethyl ester was accomplished with 2M TBAF in THF. No effort was made to acidify the product, and the resulting tetrabutylammonium carboxylate was directly coupled with *N*,*N*-dimethylproline amide. Removal of the Alloc group was carried out with $Pd(P(Ph)_3)_4$ (0.3 equiv) and phenylsilane (15 equiv) in 9/1 dichloromethane/ acetonitrile.[10] The mixed solvent system was necessary for adequate solvent detection by the ACT 357 automated synthesizer used in the library synthesis, and we found it necessary to wash the resin with 5% sodium diethylthiocarbamate in DMF (2×) to fully remove the palladium containing material. The proline amino group was then coupled with *N*-acetylproline to afford the fully derivatized scaffold. Cleavage with 90% TFA/H_2O afforded the primary carboxamide **5** in 73% overall yield. Many of the cleaved intermediates along with the final product from the solid-phase synthesis were analyzed by TLC and ^1H NMR and shown to be comparable to the same compounds prepared in solution using trimethoxybenzyl amide as a Tentagel SRAM resin surrogate.[11]



Scheme 2. Reagents and conditions: (a) Tentagel SRAM NH_2, HATU, NMM, DMF; (b) 25% pip/DMF; (c) 3-quinolinecarboxylic acid, HATU, NMM, DMF; (d) 2M TBAF, THF; (e) *N*,*N*-dimethylprolineamide, HATU, NMM, DMF; (f) $Pd(P(C_6H_5)_3)_4$, $PhSiH_3$, CH_3CN/CH_2Cl_2; (g) *N*-acetylproline, HATU, NMM, DMF; (h) 90% TFA/H_2O, 73% overall.

The library synthesis was performed on an ACT 357 MPS automated synthesizer using a manual combine/split procedure. The diversity elements are shown in Figure 1. Following the protocol outlined in Scheme 2, 12 sidechains were attached at each of the three sites to generate the 1728 member Tyr-Pro library. The ACT 357 uses a 36-well reactor block allowing us to perform a split-pool strategy that resulted in the generation of 36 mixtures of 48 compounds each. The progress of the synthesis was monitored by withdrawing small quantities of resin, approximately 10–20 mg, randomly chosen from 6 wells after addition of the first sidechain (1 compound per sample), and from a different set of 6 wells after the second coupling but before the second pooling (4 compounds per sample). These samples were cleaved with 90% TFA/H_2O and the samples analyzed by MS. In all cases the desired products were observed as the major components, and no unprotected or uncoupled products were observed. Analysis of the final products was more complicated due to the number of components in each mixture. Since the samples analyzed during the course of the library synthesis verified the successful attachment of the first two sidechains we were most interested in determining the extent of conversion for the final Alloc deprotection and amide bond formation. We investigated whether ^1H NMR could provide us with the necessary data. Twelve wells were analyzed,

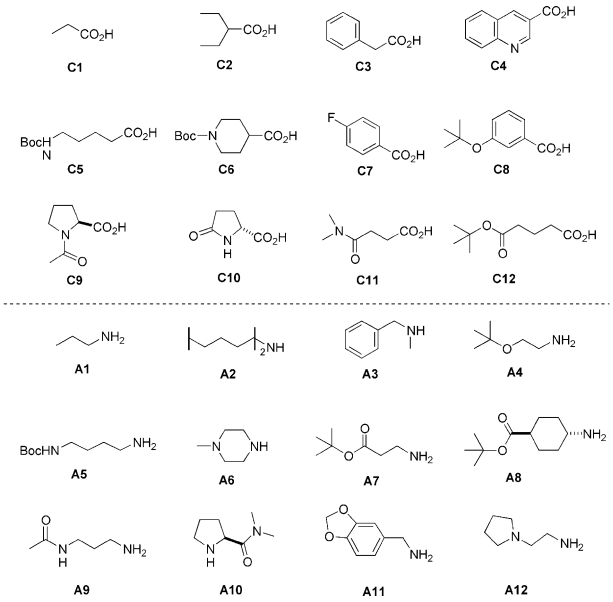


Figure 1. Carboxylic acid and amine sidechains used for the library synthesis.

and indeed in all cases there was a complete absence of alkene protons in the 5.10–5.40 ppm range indicating the palladium catalyzed deprotection of the Alloc group went to completion. Also, the absorptions due to the scaffold and to the final sidechain, which all sample members have in common, were by far the most prominent absorptions in the spectra. We therefore concluded that the library synthesis went as planned and was of high quality.

The library was screened in a functional bioassay designed to identify inhibitors of TNF-α signalling. Addition of TNF-α to A549 cells in the presence of actinomycin D causes the cells to undergo apoptosis.[12] This is a critical part of cellular homeostasis,[13] and if left unchecked results in unwanted cell death and could contribute to diseases such as sepsis or reperfusion injury.[14] TNF-α is also a pro-inflammatory cytokine, and is known to play a role in a number of inflammatory diseases such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease.[15] To determine whether the compounds could inhibit TNF-α, each of the 36 mixtures were screened in an apoptosis assay at 300 μM total final assay concentration, resulting in each individual compound being present at approximately 6 μM in the mixture.[12] The results of the assay are shown in Figure 2. Many of the pools displayed significant inhibition of apoptosis. The material derived from Pool 4 afforded the greatest inhibition, and chemical deconvolution of that well was undertaken in order to identify the active component(s). The deconvolution was carried out on the ACT 357 and we were thus able to synthesize 24 discrete compounds along with 12 mixtures of 2 compounds each. The screening data for the deconvoluted samples shows that a large percentage of the activity is due to at most four compounds (Fig. 2B, Wells 2, 14, 26). The four putative active components varied only at the tyrosine amino group, and all possessed the dipentylamide moiety at the proline carboxylate site indicating the need for a large hydrophobic group at this site. The compounds were resynthesized in solution, purified, and these screened in the apoptosis assay. Three of the four purified compounds displayed low micromolar activity in the assay (Fig. 2C). The lack of significant discrepancy between the potency of the crude library samples and the purified samples is an additional indication of the library integrity. An IC_{50} was not determined for the pooled mixture from well 26.

In summary, we have designed and synthesized a 1728 member split-pool combinatorial library based on a novel tyrosine-proline peptidomimetic scaffold. Many of the library pools were active in a TNF-α induced apoptosis assay, and through one round of chemical deconvolution of one of the pools we were able to identify compounds **7**, **8**, and **10** as structurally novel inhibitors of TNF-α signaling.

Acknowledgements

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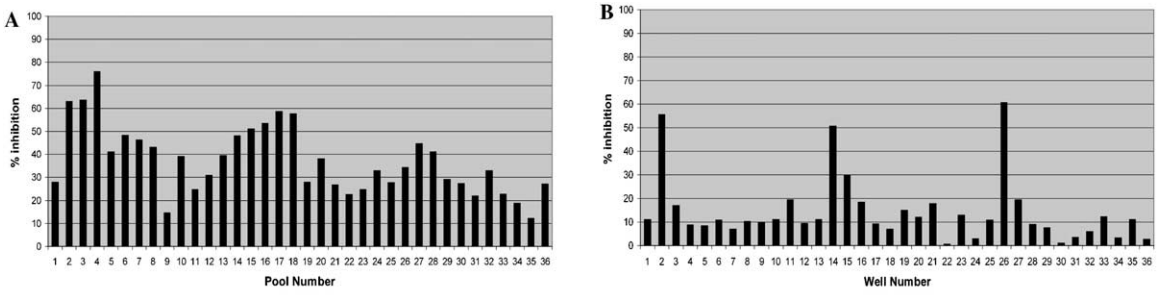

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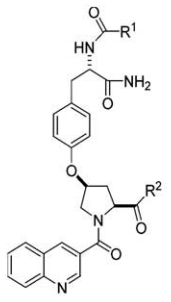
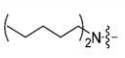
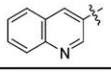
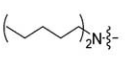
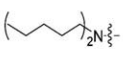
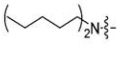


[a]NT=Not Tested

Well #	Cmpd #	R^1	R^2	IC_{50} (mM)[a] Solid phase	Resynth
2	6			11.1	8.1
14	7			15.4	12.9
26-1	8			NT	35%[b]
26-2	9			NT	10.1

[b]Percent inhibition at 20 μM

Figure 2. Screening results for the Tyr-Pro library—inhibition of TNF-α induced apoptosis: (A) Library pools 1–36, 300 μM total final assay concentration. (B) Deconvoluted compounds from Pool Number 4. Wells 1–24 are single compounds screened at 20 μM, wells 25–36 are mixtures of 2 compounds per well screened at 40μM total final assay concentration. (C) Structures of discrete active compounds and comparison of IC_{50}'s between the crude parallel synthesis material from solid phase and the resynthesized pure material.

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12. (a) Last-Barney, K.; Homon, C. A.; Faanes, R. B.; Merluzzi, V. J. *J. Immunol.* **1988**, *141*, 527. (b) Briefly, 10^5 cells in 200 μL 10% FBS/RPMI antibiotic containing culture medium were plated into 96-well round-bottom culture plates and allowed to adhere for 6 h at 37 °C in a 5% CO_2 atmosphere. The media was removed and 100 μL of RPMI + 1 μg/mL actinomycin-D was added to each well, followed by 90 μL of test compound solution in 1% DMSO. This was incubated for 1 h. TNF-α (10 μL) was added to achieve a final assay concentration equal to its EC_{50} (normally 1 ng/mL) and the plates incubated for 18 h. The media was aspirated from the plates and 100 μL of 0.5% crystal violet in 20% methanol was added. After 10 min, the plates were rinsed with water to remove excess stain, air dried, and absorbance measured at 590 nm.
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