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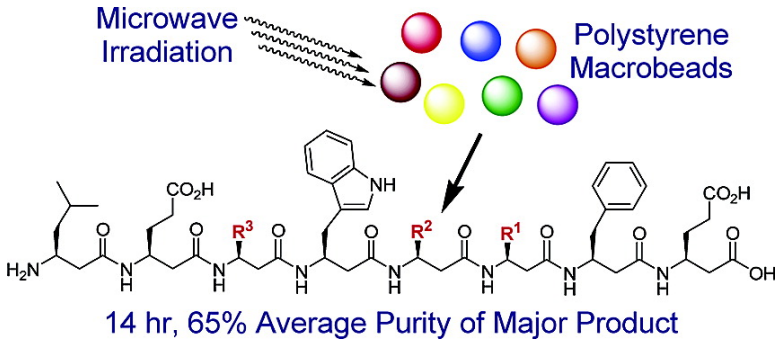

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Efficient Synthesis of a β-Peptide Combinatorial Library with Microwave Irradiation

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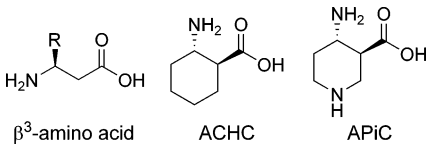
Abstract: The predictable relationship between β-amino acid sequence and folding has inspired several biological applications of β-peptides. For many such applications, it would be desirable to prepare and screen β-peptide libraries. However, standard peptide synthesis protocols are not efficient enough to support a library approach for many types of β-peptides. We recently optimized the solid-phase synthesis of β-peptides using microwave irradiation, and we have now adapted this approach to synthesis on polystyrene macrobeads. We rapidly prepared a high-quality β-peptide combinatorial library via a split-and-mix strategy. This library was screened in search of β-peptide antagonists of the p53-MDM2 protein−protein interaction.

Introduction

β-Peptides (oligomers of β-amino acids) are a well-characterized class of foldamers that can adopt a wide variety of discrete secondary structures.[1] The most intensively studied β-peptide secondary structure is the 14-helix, which is defined by 14-membered ring $N-H_i \rightarrow O=C_{i+2}$ hydrogen bonds between backbone amide groups. Seebach et al. discovered that β-peptides composed exclusively of β^3-residues can form the 14-helix,[2] and we have shown that the use of β-amino acids with a six-membered ring constraint, such as *trans*-2-aminocyclohexanecarboxylic acid (ACHC) or *trans*-4-aminopiperidine-3-carboxylic acid (APiC), leads to a dramatic enhancement in 14-helix stability relative to β^3-amino acids.[3]



β3-amino acid ACHC APiC

Combining constrained and β^3-residues allows one to prepare β-peptides that display specific arrays of diverse side chains on a stable three-dimensional scaffold.[3e] The predictable relationship between β-amino acid sequence and folding raises the prospect of endowing β-peptides with useful functions. A number of applications has been reported for β-peptides.[4] Proteolytic[5] and metabolic[6] stability and the prospect of intracellular delivery[4j] make β-peptides very attractive from a biomedical perspective.

Recent findings of Schepartz et al. are particularly interesting in the biomedical context. These workers explored the β-peptide 14-helix as a scaffold for displaying a set of side chains in a protein-mimetic manner.[4h] β-Peptides were designed to mimic the projection of three side chains from an α-helical segment of the protein p53,[7] and some of these compounds were shown to bind with moderate affinity to MDM2, which naturally binds to p53.

The discovery and optimization of bioactive 14-helical β-peptides has been hindered by the difficulty of their solid-phase synthesis.[4g,8] 14-Helical β-peptides prepared using standard solid-phase peptide synthesis (SPPS) protocols[9] are usually not of sufficient purity for direct evaluation. The necessity of

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[‡] University of South Florida.

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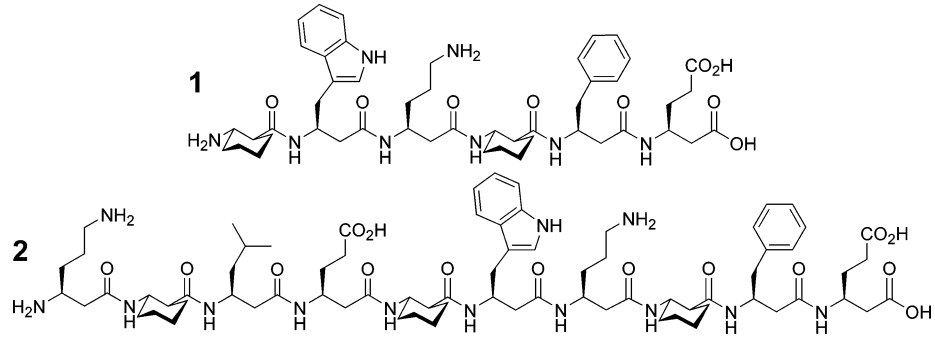

Figure 1. β-Peptides for synthetic optimization.

HPLC purification prior to screening has to date limited synthetic efforts to small sets of β-peptides. Progress toward the biological application of β-peptides could be more rapidly accomplished through the synthesis and screening of combinatorial libraries.

We have recently optimized the SPPS of 14-helical β-peptides using microwave irradiation.[10,11] During the solid-phase synthesis of these oligomers, one often observes a sudden onset of problems as the chain length reaches ca. six residues.[8] At this length, both removal of the 9-fluorenylmethoxycarbonyl (Fmoc) protecting group and amide bond formation become difficult, perhaps because of on-resin self-association of the growing β-peptide chains.[8c,12] Incorporation of ACHC tends to exacerbate these problems.[4g] We used β-peptide **1** (Figure 1) as a test sequence for synthetic optimization because of the extreme challenge of completely coupling and deprotecting the N-terminal ACHC residue.[10] Both microwave and conventional heating were found to provide β-peptide **1** in high purity, with the advantage of microwave irradiation being a 10-fold reduction in synthesis time. Microwave synthesis of the longer β-peptide **2** with a salt additive (LiCl)[13] provided the product in much higher purity than did other synthetic methods.

We used 100−200 mesh polystyrene (PS) resin (75−150 μm diameter) as the solid support for our previous synthetic optimization with the hope of identifying reaction conditions that could be applied to the generation of β-peptide combinatorial libraries on PS macrobeads using split-and-mix methods.[14] Polystyrene resin was selected because it is less expensive than is poly(ethylene glycol)-PS resin (TentaGel), although the latter is generally more effective for α- and β-peptide synthesis.[15] Also, the largest commercially available macrobeads (500−600 μm) are composed of polystyrene. The high per-bead compound loading (80−200 nmol) of this solid support is attractive for preparation of one-bead-one-stock solution libraries; each bead contains sufficient material for multiple solution-based assays as well as analytical characterization.[16] Such libraries are most useful if the compounds are generated with sufficient purity to avoid ambiguity in biological assays. Unfortunately, reaction rate decreases with increasing resin bead diameter, so library preparation on PS macrobeads can be hindered by slow reagent diffusion into the polymer matrix and sluggish reaction rates.[17] This problem is typically resolved by using extremely long reaction times and large excesses (20 equiv) of reagents, neither of which is attractive.[18] As microwave irradiation has been found to reduce synthesis time,[10] we have applied this method to reactions on PS macrobeads to address the inherent limitations of this solid support.

Results and Discussion

β-Peptide Synthesis on PS Macrobeads. We sought to optimize synthesis on PS macrobeads as the next step toward the preparation of β-peptide combinatorial libraries. The high cost of Fmoc-β-amino acids makes it preferable to use as few monomer equivalents per coupling as possible; we employed 3 equiv. Manual synthesis of β-peptides **1** and **2** on PS macrobeads under extended reaction conditions at room temperature (6 h per amide bond forming reaction and 1 h per Fmoc removal reaction) provided products with 69 and 56% purity, respectively (Figure 2). The purities were improved to 87 and 70% by heating the coupling and deprotection reactions to 65 °C in an oil bath (Figure 2, Oil Bath A), but the 42 and 77 h overall synthesis times were cumbersome.

Blackwell has shrewdly observed that microwave irradiation could be used to accelerate reactions on PS macrobeads, thus overcoming the limitation of having to use long reaction times.[19] At first, it was unclear to us how to adapt the rapid heating of microwave irradiation to synthesis on PS macrobeads. Our initial attempts were only moderately successful: direct application of our microwave-assisted SPPS conditions (4 min deprotection and 6 min coupling reactions) developed for 100−200 mesh PS resin to the synthesis of β-peptide **1** on PS macrobeads provided the product in extremely low yield (data not shown). We suspected that only the surface of the resin beads was being functionalized, because the reagents were not likely to have diffused very deeply into the polymer matrix during the short

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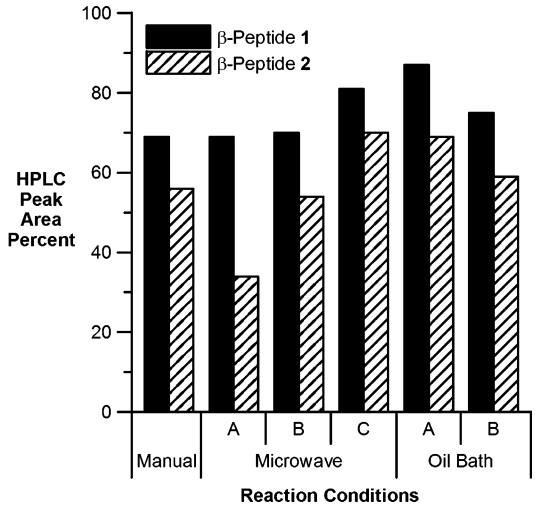

Figure 2. Initial purity of β-peptides **1** and **2** (peak area percent, from analytical reverse-phase (RP) HPLC monitored via UV absorbance at 220 nm) from synthesis on PS macrobeads under different conditions. All coupling and deprotection reactions were conducted under the given reaction condition (i.e., manual, microwave, or oil bath) as described below. ACHC-1 in β-peptide **1** and ACHC-2 and ACHC-5 in β-peptide **2** were double-coupled and -deprotected in all syntheses. The coupling of these residues was performed in 0.8 M LiCl in NMP in the microwave and oil bath syntheses; all other coupling reactions were performed in DMF. Maximum power for all microwave reactions was 50 W. Microwave-assisted deprotections were performed at 60 °C, and couplings were carried out at 50 °C in DMF or 45 °C in 0.8 M LiCl in NMP. Manual: 1 h deprotection, 6 h coupling, RT; Microwave A: 1 h deprotection at RT followed by 2 min ramp and 2 min hold, 2 h coupling at RT followed by 2 min ramp and 4 min hold; Microwave B: 2 min ramp and 1 h hold for deprotection, 2 min ramp and 1 h hold for coupling; Microwave C: 3 × (2 min ramp followed by 10 min cool at RT) for deprotection, 6 × (2 min ramp followed by 10 min cool at RT) for coupling; Oil Bath A: 1 h deprotection, 6 h coupling, 65 °C; and Oil Bath B: 30 min deprotection, 1 h coupling, 65 °C.

reaction time. Allowing the coupling to proceed for 2 h at room temperature while the reagents diffused into the macrobeads, followed by our typical 6 min microwave irradiation, gave β-peptide **1** in 69% purity and β-peptide **2** in 34% purity (Figure 2, Microwave A). Only marginal enhancement was obtained by performing each reaction with 1 h of continuous microwave irradiation (70% purity for **1** and 54% for **2**; Figure 2, Microwave B). (After the initial 2 min ramp to the desired temperature, the power input was modulated to maintain that temperature, resulting in a very low level of microwave irradiation (<5 W) being employed for a majority of the reaction time.) However, significant improvement was achieved by repeating a cycle of 2 min of microwave irradiation to reach the desired temperature followed by 10 min of cooling (6 cycles per coupling, 3 cycles per deprotection). Subjecting the sample to several short bursts of intense microwave irradiation was found to be more effective than using a continuous but lower level of irradiation, as the former method provided β-peptide **1** on PS macrobeads in high purity (81%). The reaction time was significantly reduced relative to oil bath heating (9 vs 42 h). β-Peptide **2** was produced in 70% purity in only 16 h under these conditions (Figure 2, Microwave C). Thus, we observed a reduced reaction time for microwave irradiation in comparison with conventional heating, but the two methods gave comparable purities for both β-peptides.

To ascertain whether microwave irradiation gives a rate enhancement for synthesis on PS macrobeads, we prepared β-peptides **1** and **2** using oil bath heating for short times (1 h coupling, 30 min deprotection). We found that both **1** and **2** were produced in slightly higher purities using conventional heating (75 and 59%; Figure 2, Oil Bath B) relative to continuous microwave irradiation for the same reaction time (70 and 54%; Figure 2, Microwave B). However, in neither case do the results match those obtained using multiple cycles of microwave irradiation (81 and 70%; Figure 2, Microwave C), which also require only 1 h per coupling and 30 min per deprotection reaction. Therefore, our optimized microwave irradiation conditions provide a moderate advantage for synthesis on PS macrobeads, relative to the other conditions examined, as the optimized conditions provide the product in the highest purity and shortest synthesis time. The temporal advantage is especially important for oligomeric molecules, such as β-peptides, that require many sequential reactions.

In our previous study of β-peptide solid-phase synthesis on a different resin (PS 100−200 mesh), we found that both conventional heating and microwave irradiation gave similar results for the shorter β-peptide **1** if reactions were heated 10 times longer in the oil bath than in the microwave reactor.[10] However, synthesis of the longer β-peptide **2** with microwave irradiation had shown a significant advantage over conventional heating (beyond the decrease in time). We had speculated that the microwave advantage for the synthesis of **2** reflects the increasing difficulty of couplings and deprotections after the fifth residue, which may arise from aggregation and/or folding during growth of the β-peptide chain. Perhaps a longer period of conventional heating for each coupling/deprotection cycle for the final five residues would eventually have allowed us to produce β-peptide **2** in purity comparable to that of the microwave synthesis, as was the case for β-peptide **1**. An increasing number of reports suggests that if microwave and conventional heating reactions are carried out under identical conditions, then the results obtained with both methods will be the same (i.e., there is no nonthermal microwave effect).[20] Although we went to great lengths to carry out reactions under very similar experimental conditions (temperature, atmospheric pressure, agitation with N_2 bubbling) for both methods of energy input, we (and others[21]) found that conventional heating is unable to duplicate the rapid internal heating provided by microwave irradiation. (Heating a coupling solution of DMF from room temperature to 50 °C with <50 W of microwave irradiation requires ∼1.5 min; the same process takes ∼4 min in an oil bath.) We previously found also that the salt additive accentuates the rapid heating and thus the advantage of microwave synthesis for β-peptide **2**.[10] (Heating a coupling solution of 0.8 M LiCl in NMP from room temperature to 50 °C with <50 W of microwave irradiation requires <1 min.) Synthesis on PS macrobeads presents a new challenge because the effectiveness of rapid microwave heating is limited since the short irradiation cycle is finished before the activated monomer can diffuse throughout the polymer matrix and react with the free N-termini of the growing peptide chains. Employing a continuous, low level of microwave irradiation seems to exert a purely thermal effect, as synthesis under these conditions (Microwave B) gives results similar to those obtained with oil bath heating for short times (Oil Bath B). Therefore, the previously observed beneficial effects, obtained with 100−200

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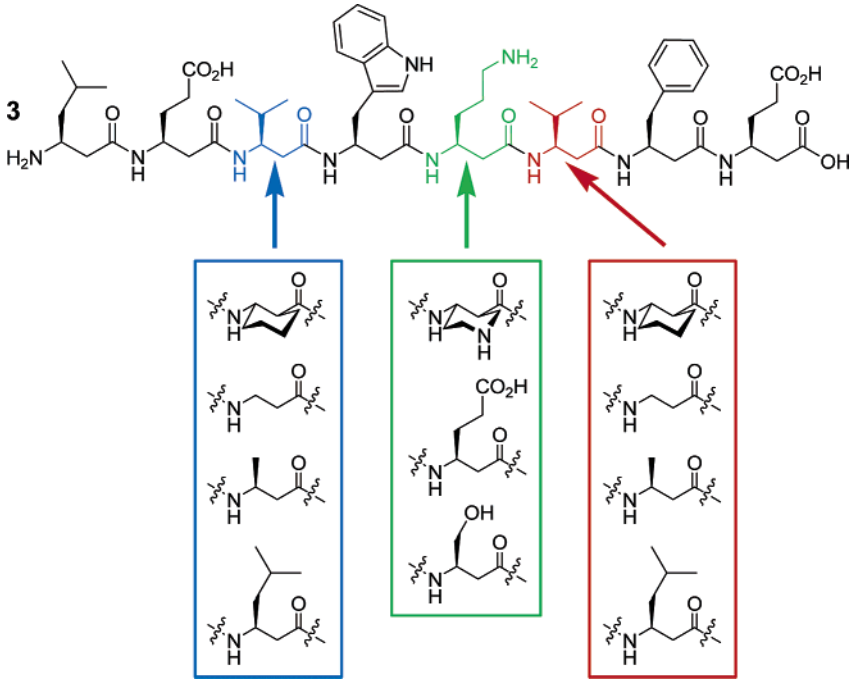

Figure 3. Octa-β-peptide library produced via split-and-pool synthesis on PS macrobeads with microwave irradiation. Five different residues were incorporated at positions 3 and 6; four different residues were installed at position 5 ($5 \times 4 \times 5 = 100$ members). The sequence of β-peptide **3** is from ref 4h.

mesh PS resin as a result of rapid internal heating with microwave irradiation,[10] are partially abrogated by slow reagent diffusion and the long reaction times required with PS macrobeads. However, using multiple cycles of irradiation (Microwave C) reestablishes the advantage of microwave heating by reducing the synthesis time needed to produce β-peptide **2** in similar purity (~70%) to that achieved with conventional heating for longer periods of time (Oil Bath A). Perhaps the rapid heating associated with intense microwave irradiation is able to disrupt on-bead β-peptide self-association more effectively than does conventional heating or continuous lower levels of microwave irradiation, thus driving the reaction to completion more quickly.[22]

Synthesis and Characterization of a β-Peptide Combinatorial Library. Following our synthetic optimization on PS macrobeads, we used microwave irradiation for rapid generation of a 100-member β-peptide library via the split-and-pool technique (Figure 3). This library was based on octa-β-peptide **3**, which was reported to block the interaction between the protein MDM2 and a 17-residue peptide from the N-terminal region of p53.[4h] The tumor suppressor protein p53 is a transcription factor that controls cellular response to stress through the induction of cell cycle arrest or apoptosis.[23] MDM2 regulates p53 activity by binding to the α-helical transactivation domain near the N-terminus of p53, thereby targeting p53 for proteosomal degradation.[24] Overexpression of MDM2 inhibits activation of the p53 pathway, leading to uncontrolled cell proliferation; MDM2 overexpression is commonly observed in soft-tissue tumors, osteosarcomas, and esophageal carcinomas.[25] Disruption of the p53-MDM2 interaction is therefore a therapeutic target for the treatment of cancer.[26] Extensive efforts toward this end have yielded tightly binding α-peptides and potent, bioactive small molecule antagonists of the p53-MDM2 interaction.[27]

Octa-β-peptide **3** was reported to inhibit the interaction between MDM2 and a fluorescently labeled α-peptide corresponding to residues 15−31 of wild-type p53 with a median inhibitory concentration (IC_{50}) of approximately 80 μM in a fluorescence polarization (FP) competition assay.[4h] β-Peptide **3** was designed to adopt a 14-helical conformation that displays three critical side chains, those of β^3-hLeu-1, β^3-hTrp-4, and β^3-hPhe-7, along one side of the helix.[7] These β-peptide side chains are intended to mimic those of Leu-26, Trp-23, and Phe-19 of p53, which are observed to align along one side of a distorted α-helix in a complex between MDM2 and an α-peptide corresponding to residues 15−29 of p53.[28] Since the unlabeled α-peptide corresponding to residues 15−31 had an IC_{50} of ca. 2 μM in the FP competition assay, roughly 40-fold lower than the IC_{50} of β-peptide **3**, we wondered whether a combinatorial variation of the sequence of **3** would generate improved inhibitors. Our library was designed to probe the effect of conformational stability on binding affinity by incorporating both structure-promoting (ACHC) and structure-destabilizing residues (β-hGly) at positions 3 and 6. The degree of hydrophobicity at these positions was varied by including β^3-hLeu,

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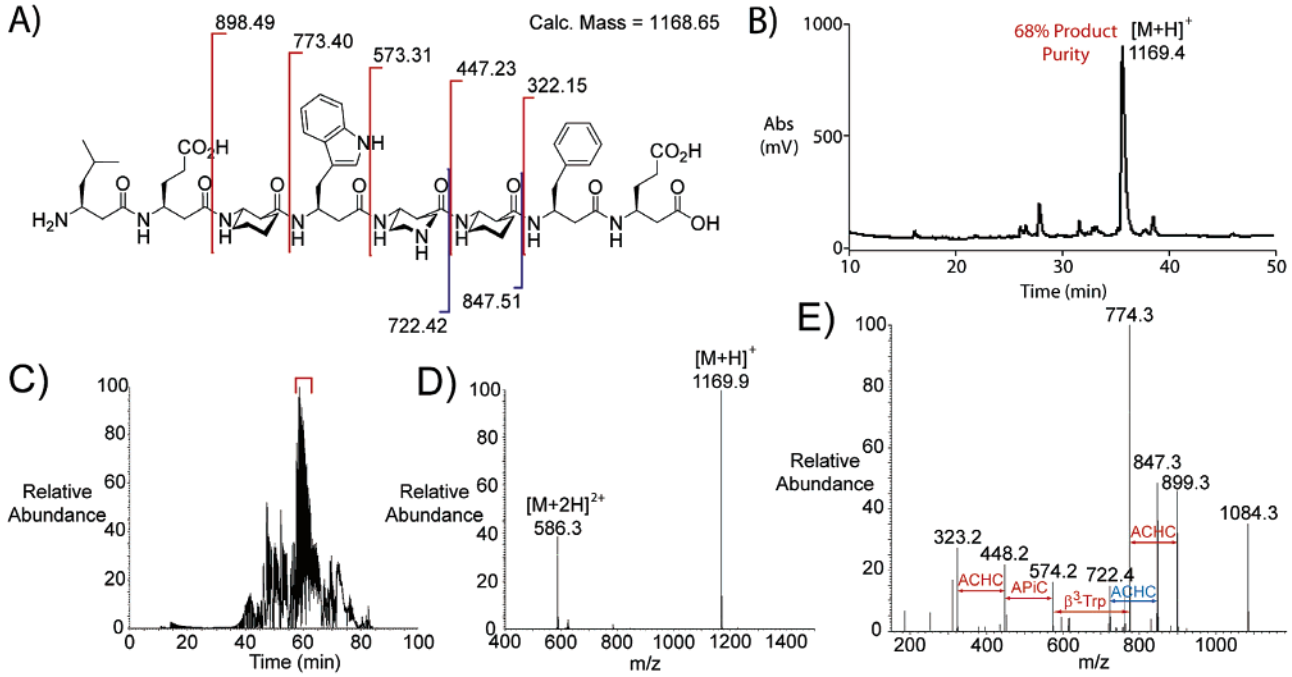

Figure 4. Characterization a β-peptide library member. (A) Deduced library member from MW and observed fragmentation pattern (calculated masses). (B) Analytical RP-HPLC trace (68% product purity) with MW from MALDI-TOF MS of major peak. (C) Base peak chromatogram of μLC (monitored by total ion count of most abundant ion in each scan). (D) ESI-MS of bracketed region (57.5−62 min) in panel C. (E) ESI-MS/MS of $[M + 2H]^{2+}$ parent ion in panel D with assigned β-amino acid fragments.

β^3-hVal, and β^3-hAla as well. We also varied the nature of the side chain at position 5: replacing β^3-hOrn with β^3-hSer or β^3-hGlu may better mimic the side chains displayed at this position in the p53 α-helix, according to superimposition analysis.

We constructed the library on PS macrobeads in two days with multiple cycles of microwave irradiation. Reaction progress was monitored using 2,4,6-trinitrobenzenesulfonic acid (TNBS), which stains beads containing unreacted amino groups.[8c] Incomplete coupling of β^3-hPhe-7 was detected at the end of the standard reaction cycle, so the mixture was subjected to an additional three microwave irradiation/cool cycles. The APiC residue at position 5 was double-coupled in DMF; ACHC-3 was double-coupled in NMP/LiCl.[10] All other residues at position 3 were double-coupled in DMF.

To assess the quality of the library, 50 beads were selected at random, and the material from them was characterized. In all cases, the major peak in the analytical RP-HPLC trace corresponded to the molecular weight of an expected library member, which indicates an excellent library quality.[29] β-Peptides from 10 of the randomly chosen beads were sequenced by μLC-MS/MS.[30] In each case, the deduced sequence corresponded to an expected member of the library, with a matching parent molecular weight. We were especially pleased that the sequence containing three cyclically constrained residues (Figure 4A), expected to be the most difficult library member to synthesize, was found among the 50 sequences via identification of its unique molecular weight. This β-peptide was produced in an acceptable 68% purity (Figure 4B), and sequencing confirmed its identity (Figure 4C−E). The average purity of

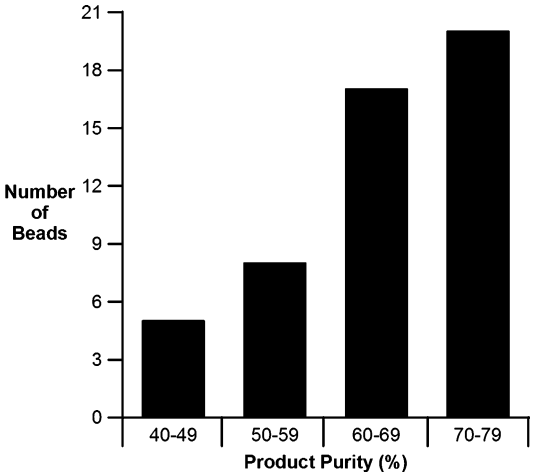

Figure 5. Product purity of material from 50 randomly selected beads from the octa-β-peptide library, determined as the area percent of the major peak in the analytical RP-HPLC chromatogram (UV absorbance at 220 nm).

β-peptides from the library was 65% (Figure 5).[31] This high level of initial purity raises confidence in screening results obtained with initial products. This effort represents the first β-peptide combinatorial library and, to our knowledge, the first example of a split-and-pool synthesis on PS macrobeads with microwave irradiation.[32,33]

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(31) The major impurity from each bead had an average of only 10% of the total HPLC peak area. The lowest purity library members were still readily identified through μLC-MS/MS sequencing and were each found to contain β^3-hSer at position 5. However, the major impurities were not simply β^3-hSer deletion sequences but were quite varied among the product mixtures from different beads.
(32) A peptoid combinatorial library was synthesized on TentaGel macrobeads using microwave irradiation: Alluri, P. G.; Reddy, M. M.; Bachhawat-Sikder, K.; Olivos, H. J.; Kodadek, T. *J. Am. Chem. Soc.* **2003**, *125*, 13995.
(33) Most microwave-assisted syntheses of combinatorial libraries have been performed in parallel using automated sequential irradiation: Kappe, C. O. *Angew. Chem., Int. Ed.* **2004**, *43*, 6250.

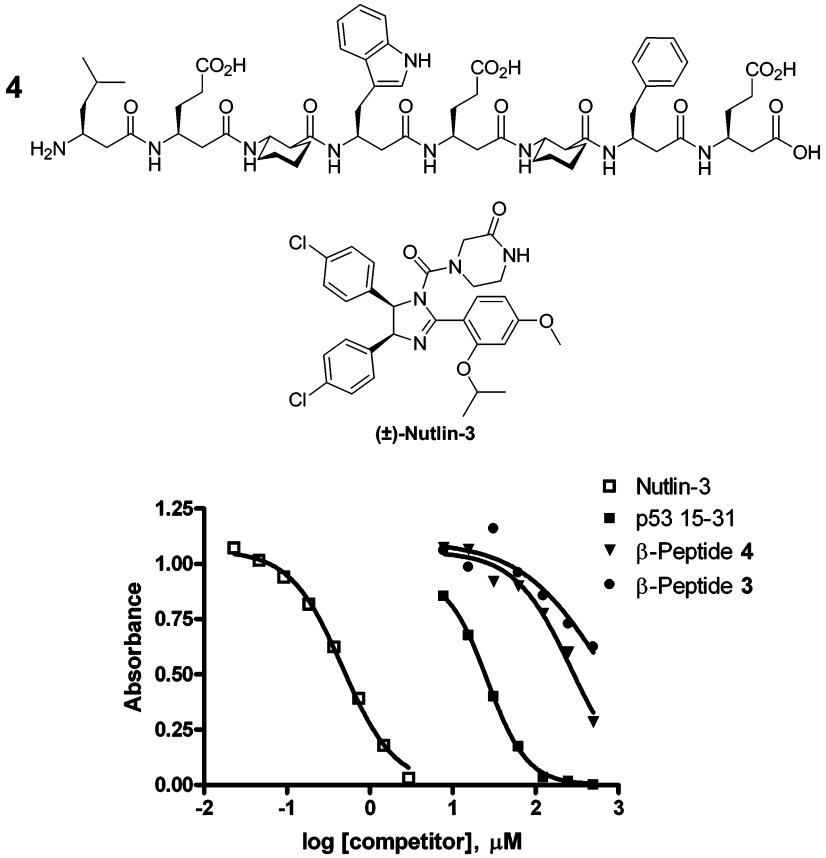

Figure 6. Binding data from the competition ELISA fitted with a one-site competition binding model (GraphPad Prism 4.0).

Library Screening for Inhibition of the p53-MDM2 Interaction. We tested the library in a competition ELISA to determine whether any of the members was able to inhibit p53-MDM2 association with greater potency than β-peptide **3**. This assay format differs from the FP format used previously[4h] because the ELISA tests for the inhibition of a protein−protein interaction, while the FP assay reports on inhibition of a protein−peptide interaction. Plates were coated with full-length human p53 bearing a His_6 tag. Crude β-peptide from a single macrobead and full-length human MDM2 fused to GST were added to each well. Screening at an approximate β-peptide concentration of 75 μM identified seven library members exhibiting roughly 80% inhibition, while β-peptide **3** showed only about 15% inhibition at this concentration (see Supporting Information). The hits from the library were identified via mass spectrometric sequencing, revealing seven different sequences (Table 1).[34] These seven β-peptides were then individually resynthesized using our microwave conditions,[10] purified by HPLC, and reevaluated in the ELISA (see Supporting Information). No significant variation in activity was observed among these seven β-peptides, apparently because the assay is relatively insensitive for such weakly binding compounds. Octamer **4** was selected for more detailed examination.

Table 1. Octa-β-peptide Hits from Library Screening and Sequencing

1	2	3	4	5	6	7	8	
H	β^3-hLeu	β^3-hGlu	ACHC	β^3-hTrp	β^3-hGlu	ACHC	β^3-hPhe	β^3-hGlu OH
H	β^3-hLeu	β^3-hGlu	ACHC	β^3-hTrp	β^3-hSer	ACHC	β^3-hPhe	β^3-hGlu OH
H	β^3-hLeu	β^3-hGlu	ACHC	β^3-hTrp	β^3-hGlu	β^3-hLeu	β^3-hPhe	β^3-hGlu OH
H	β^3-hLeu	β^3-hGlu	β^3-hLeu	β^3-hTrp	β^3-hGlu	β^3-hLeu	β^3-hPhe	β^3-hGlu OH
H	β^3-hLeu	β^3-hGlu	β^3-hLeu	β^3-hTrp	β^3-hSer	β^3-hLeu	β^3-hPhe	β^3-hGlu OH
H	β^3-hLeu	β^3-hGlu	β^3-hVal	β^3-hTrp	β^3-hSer	β^3-hAla	β^3-hPhe	β^3-hGlu OH
H	β^3-hLeu	β^3-hGlu	β^3-hVal	β^3-hTrp	β^3-hOrn	β^3-hGly	β^3-hPhe	β^3-hGlu OH

Library member **4** displayed an IC_{50} value of ca. 250 μM (Figure 6); it is slightly more effective than the original β-peptide **3** (IC_{50} > 600 μM). β-Peptide **4** contains ACHC residues at positions 2 and 6, which should strongly promote the 14-helical conformation. However, stabilizing the 14-helix does not seem to provide a benefit in terms of interfering with p53-MDM2 binding because other β-peptides among the seven active library members contain β^3-hLeu in place of one ACHC or both, or even β-hGly at one of these positions. These findings suggest that the β-peptide 14-helix may not be an ideal scaffold for the development of p53-MDM2 antagonists. Indeed, molecular modeling with fixed backbone geometry indicates that the 14-helix is considerably wider than is the α-helix, the secondary structure adopted by the N-terminal segment of p53 when bound to MDM2 (see Supporting Information). Schepartz and co-workers[7] have suggested that β-peptides composed entirely of β^3-residues can depart slightly from an idealized 14-helical conformation, as expected based on the well-known flexibility

(34) A reviewer asked whether any hits were redundantly identified; none was. Statistically, using five beads per compound ensures adequate coverage of this small library (between 95 and 99% of the theoretical library members will be represented) but does not guarantee that there will be multiple copies of the same compound within the library. Furthermore, the removal of 50 beads for quality control, bead breakage during synthesis (reducing the yield), an arbitrary definition of what level of percent inhibition constitutes a hit, and the low sensitivity of the assay to weakly binding compounds all potentially explain why multiple copies of the same compound were not identified. Since the identified compounds do show slightly improved activity, they were validated as hits and not false positives. Burgess, K.; Liaw, A. I.; Wang, N. *J. Med. Chem.* **1994**, *37*, 2985.

and low intrinsic 14-helical propensity of β^3-residues,[35] and they have speculated that such distortion is necessary for optimal binding to the MDM2 cleft. The contrast we observe between the lack of significant variation in activity among the hits from our library and the wide range of expected 14-helical propensities among these compounds (arising from variations in ACHC and β-hGly content) raises the possibility that small conformational distortions are not important for MDM2 affinity, at least among the weakly binding β-peptides reported to date.

Six of the seven active library members contained a replacement for β^3-hOrn, the original residue at position 5. The β^3-hOrn side chain should be cationic under assay conditions; a positive charge at this position was dictated in the original design[4h] by the need for side chain ion pairing to stabilize the 14-helical conformation. We explored the effect of anionic (β^3-hGlu) and neutral (β^3-hSer) side chains at this position in the library, which may better mimic the Ser-20 and Asp-21 residues in the p53-derived α-peptide. Although molecular modeling suggested to us that the β-amino acid side chain at position 5 is solvent-exposed, the results imply that such replacements of β^3-hOrn-5 are at least modestly beneficial for β-peptide binding to MDM2.

Included in our ELISA as a positive control for inhibition of the p53-MDM2 interaction was a p53-derived α-peptide comprising residues 15−31. This α-peptide contains all three hydrophobic residues that are essential for binding to MDM2 (Leu-26, Trp-23, and Phe-19) and has an IC_{50} of ca. 25 μM in our ELISA, which is consistent with prior results.[27a,b] Thus, although library member **4** is marginally improved relative to the original β-peptide sequence, **3**, these β-peptides are considerably less effective than the natural α-peptide sequence 15−31; comparable findings were reported by Schepartz et al. based on FP competition assays.[4h]

As a further positive control, we examined Nutlin-3, a synthetic small molecule that effectively inhibits the p53-MDM2 interaction in vitro and is orally active as an anticancer agent in vivo.[27c] We measured an IC_{50} of ca. 0.5 μM, which is consistent with the original report.[27c] This small molecule is superior as an inhibitor of p53-MDM2 binding relative to all β-peptides described here.

Conclusion

We have shown that recent improvements in the solid-phase synthesis of β-peptides using microwave irradiation and a salt additive can be extended to synthesis on PS macrobeads. Relative to conventional methods, the microwave approach significantly reduces synthesis time and amounts of reagents, which until now have been major limitations on the use of PS macrobeads to generate one-bead-one-stock solution combinatorial libraries. Microwave irradiation allowed rapid synthesis of a high-purity β-peptide library via a split-and-mix approach. This library was screened for inhibitors of the p53-MDM2 interaction. Hits from this library provided marginal improvement relative to a previously reported β-peptide sequence. Our failure to find a more potent inhibitor in this library may reflect the small size of our library (insufficient diversity), or this result may indicate that the 14-helix is not well-suited to mimic the α-helical segment of p53. In a search for inhibitors of a different α-helix/cleft interaction, the binding of a BH3 domain to Bcl-x_L, we have found β-peptide helices to be unproductive scaffolds, but alternative foldamers containing both α- and β-amino acid residues lead to effective inhibitors.[36] Ultimately, β-peptides and other foldamers may be best suited to disruption of protein−protein interactions that involve flatter surfaces on each partner than are found in the p53-MDM2 pair. Flat interface architectures appear to be particularly challenging for low molecular weight antagonists.[37] The methodological developments reported here will facilitate combinatorial exploration of β-peptides and other foldamers as scaffolds for the discovery of protein−protein interaction inhibitors.

Experimental Procedures

General Procedures. Fmoc-(*S*,*S*)-ACHC-OH and Fmoc-(*S*,*S*)-APiC-(Boc)-OH were prepared by the method of Schinnerl et al.[3d] Fmoc-β^3-amino acids were prepared from their corresponding Fmoc-L-α-amino acids (Novabiochem) as described previously.[8a] 1-Methyl-2-pyrrolidinone (NMP) was purchased from Advanced ChemTech. Methanol, CH_2Cl_2, and acetonitrile were purchased from Burdick and Jackson. 1-Methylimidazole, piperidine, 1-hydroxybenzotriazole hydrate (HOBt), *i*Pr_2EtN (DIEA), trifluoroacetic acid (TFA), triethylsilane (TES), and DMSO were purchased from Aldrich. 1-(2-Mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT), *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), PS Wang resin (100−200 mesh), and NovaSyn TGR resin were purchased from Novabiochem. Polystyrene A PHB resin (500−560 μm macrobeads) was purchased from Rapp Polymere. DMF (biotech grade solvent, 99.9+%) was purchased from Aldrich and stored over Dowex ion-exchange resin. 2,4,6-Trinitrobenzenesulfonic acid (TNBS, 1% solution in DMF) was purchased from Fluka. Nutlin-3 ((±)-4-[4,5-bis-(4-chlorophenyl)-2-(2-isopropanoxy-4-methoxy-phenyl)-4,5-dihydro-imidazole-1-carbonyl]-piperazin-2-one) was purchased from Cayman Chemical. Dry CH_2Cl_2 and *i*Pr_2EtN were distilled from calcium hydride.

First Residue Loading. First residue loading was accomplished as described with some modifications.[8c,38] Fmoc-(*S*)-β^3-hGlu(*t*Bu)-OH (0.824 g) was activated with 1-methylimidazole (112 μL) and MSNT (556 mg) in dry CH_2Cl_2 (5.6 mL) and added to DMF-swollen Polystyrene A PHB macrobeads (500 mg, 500−560 μm resin, 0.75 mmol/g initial loading, Rapp Polymere) in a polypropylene solid-phase extraction (SPE) tube (25 mL, Alltech). The tube was capped and placed on a wrist-action shaker (Labquake, Barnstead/Thermolyne). After reaction for 12 h at room temperature, the resin was washed (5 × CH_2Cl_2, 5 × DMF, 5 × CH_2Cl_2, and 5 × MeOH) using a vacuum manifold (Vac-Man, Promega) connected to a water aspirator and then dried under a stream of N_2 until it was free-flowing. The yield was estimated by UV quantification of the dibenzofulvene-piperidine adduct at 290 nm as previously described (0.48 mmol/g, 64%).[39]

Microwave β-Peptide Synthesis on PS Macrobeads Using Ramp/Cool Cycles. Fmoc-β^3-hGlu(*t*Bu)-loaded PS macrobeads (10 μmol, 21 mg) were placed in a modified polypropylene SPE tube (4 mL, Alltech, top rim removed with a razor blade) and swelled with DMF for approximately 10 min. The resin was washed (5 × DMF, 5 × CH_2Cl_2, and 5 × DMF). A deprotection solution (750 μL of 20% piperidine in DMF (v/v)) was added to the resin, and the tube was placed inside a glass 10 mL microwave reaction vessel containing ∼2 mL of DMF (Figure 7). A N_2 line was inserted for agitation, and the vessel was placed in the microwave reactor (CEM Discover) and irradiated (50 W maximum power, 60 °C, ramp 2 min). The sample was then cooled

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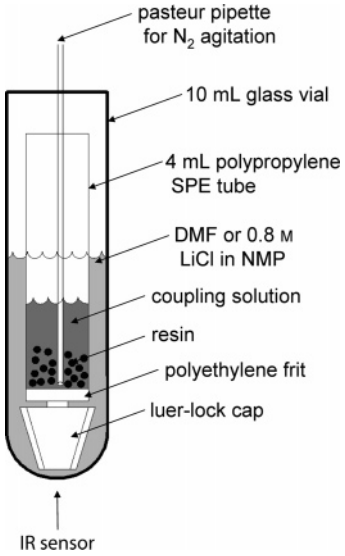

Figure 7. Experimental setup for small-scale microwave SPPS of β-peptides (SPE = solid-phase extraction).

with a stream of compressed air for 10 min. This ramp/cool cycle was repeated a total of 3 times for deprotection. The tube was removed from the microwave reactor, and the resin was washed as before. In a separate vial, Fmoc-β-amino acid (30 μmol) was activated by adding HBTU (60 μL of 0.5 M solution in DMF), DMF (440 μL), HOBt (60 μL of 0.5 M solution in DMF), and iPr$_2$EtN (60 μL of 1.0 M solution in DMF). The mixture was vortexed and added to the resin. The sample was irradiated in the microwave reactor (50 W maximum power, 50 °C, ramp 2 min). The sample was then cooled for 10 min; this ramp/cool cycle was repeated a total of 6 times per coupling step. Alternatively, the coupling of Fmoc-(*S*,*S*)-ACHC-OH was performed by activating with solutions of HBTU, HOBt, and iPr$_2$EtN in NMP and adding a solution of LiCl in NMP for a final concentration of 0.8 M LiCl (620 μL final volume) and then adding this solution to the resin. The tube was placed in a microwave reaction vessel containing approximately 2 mL of 0.8 M LiCl in NMP and irradiated (50 W maximum power, 45 °C, 6 ramp/cool cycles). (The target temperature was set to only 45 °C, but the higher ionic strength of the 0.8 M LiCl in NMP solution results in a greater efficiency of energy transfer, so the final temperature is similar to that observed for a coupling reaction in DMF set at 50 °C. Using our reaction vessel with the IR temperature sensor gave reproducible results with the empirically derived set temperatures, but the temperature measurements were not accurate.[40]) After the coupling reaction, the resin was washed as before. The N-terminal ACHC residue in β-peptide **1** and ACHC-2 and ACHC-5 in β-peptide **2** were double-coupled and -deprotected. All other couplings were performed once with DMF as the solvent. The deprotection/coupling cycle was repeated in a stepwise manner until reaching the desired length of the hexamer or decamer.

Manual β-Peptide Synthesis on PS Macrobeads. Loaded PS macrobeads (10 μmol, 21 mg) were placed in an SPE tube (4 mL, Alltech) and swelled with DMF. The resin was washed (5 × DMF, 5 × CH_2Cl_2, and 5 × DMF). A deprotection solution was added to the resin, and the tube was capped and placed on a shaker for 1 h at room temperature. The tube was removed from the shaker, and the resin was washed as before. A solution of activated Fmoc-β-amino acid was added to the resin, and the tube was capped and placed on the shaker for 6 h at room temperature. After the coupling reaction, the resin was washed as before. The deprotection/coupling cycle was repeated in a stepwise manner until reaching the desired length of the hexamer or decamer. All coupling reactions were performed in DMF. The N-terminal ACHC residue in β-peptide **1** and ACHC-2 and ACHC-5 in β-peptide **2** were double-coupled and -deprotected.

Oil Bath β-Peptide Synthesis on PS Macrobeads. Loaded PS macrobeads (10 μmol, 21 mg) were placed in a SPE tube (4 mL, Alltech) and swelled with DMF. The resin was washed (5 × DMF, 5 × CH_2Cl_2, and 5 × DMF). A deprotection solution was added to the resin, a N_2 line was inserted for agitation, and the tube placed in an oil bath at 65 °C for 1 h. The tube was removed from the oil bath, and the resin was washed as before. A solution of activated Fmoc-β-amino acid was added to the resin, a N_2 line was inserted for agitation, and the tube was placed in an oil bath at 65 °C for 6 h. After the coupling reaction, the resin was washed as before. The deprotection/coupling cycle was repeated in a stepwise manner until reaching the desired length of the hexamer or decamer. The N-terminal ACHC residue in β-peptide **1** and ACHC-2 and ACHC-5 in β-peptide **2** were double-coupled in 0.8 M LiCl in NMP and double-deprotected. All other coupling reactions were performed once in DMF.

β-Peptide Cleavage and HPLC Analysis. After the final residue had been added and deprotected, the resin was washed (5 × DMF, 5 × CH_2Cl_2, 5 × DMF, and 5 × CH_2Cl_2), and the β-peptide was cleaved from the solid support with simultaneous side chain deprotection (3 mL, 45:45:5:5 TFA/CH_2Cl_2/TES/water, 2 h, RT, with rocking). The cleavage solution was drained and concentrated under a stream of N_2. The crude β-peptide mixture was dissolved in 1.0 mL of DMSO, diluted with DMSO (1−20), and analyzed by HPLC (10 μL injection, Shimadzu). The compound was eluted from a C_4-silica reverse-phase analytical column (5 μm, 4 mm × 250 mm, Vydac) with a gradient of acetonitrile in water (10−60%, 50 min, 0.1% TFA in each) at a flow rate of 1 mL/min. The product purity was determined as the peak area percent by integration of the UV absorbance at 220 nm. Integration was performed over the 15−50 min time interval to exclude the large absorbance of DMSO that elutes from 5 to 15 min. The lower threshold of integration was set to exclude minor peaks whose areas were <1% of the peak area of the major species. β-Peptide masses were measured by MALDI-TOF-MS (Bruker Reflex II, α-cyano-4-hydroxycinnamic acid matrix).

Calculation of β-Peptide 3 Yield from a Single Macrobead. Yield from the synthesis of β-peptide **3** on PS macrobeads was quantified by the method of Yan et al.[41] β-Peptide **3** was synthesized on PS macrobeads on a 10 μmol scale with microwave irradiation as described previously. Material was cleaved from a portion of the beads and analyzed by HPLC as stated previously. The product had a purity of 64%. This sample of crude β-peptide **3** was purified by C_4-silica preparative reverse-phase HPLC. The compound was eluted from the column (10 μm, 22 mm × 250 mm, Vydac) with a gradient of acetonitrile in water (24−54%, 30 min, 0.1% TFA in each) at a flow rate of 15 mL/min. After lyophilization, a small sample (~1 mg) of purified β-peptide **3** was dissolved in 1.0 mL of DMSO to make a 0.53 mM stock solution, the concentration being determined by β^3-hTrp absorbance in 6 M guanidine hydrochloride.[42] The stock solution was then diluted to make a series of calibration solutions containing a fixed concentration of Fmoc-Phe-OH (retention time = 40 min) as an external standard to compensate for instrumental fluctuation and other systematic errors. These solutions were analyzed by analytical RP-HPLC (C_4-silica reverse-phase analytical column (5 μm, 4 mm × 250 mm, Vydac) eluted with a gradient of acetonitrile in water, 10−60%, 50 min, 0.1% TFA in each, at a flow rate of 1 mL/min) and monitored by UV absorbance at 220 nm. The peak area of β-peptide **3** was divided by the peak area of the external standard to give the peak area ratio [peak area ratio = $(\text{peak area})_{\beta-\text{peptide3}}/(\text{peak area})_{\text{externalstandard}}$]. A plot of the peak area ratio versus concentration yielded a calibration curve (see Supporting Information). The curve was fit by linear regression

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[peak area ratio = 10.56(concentration) − 0.0449] with a correlation coefficient (R^2) of 0.9921. The calibration curve was validated by preparing a solution of β-peptide **3** of a known concentration and analyzing by HPLC with UV detection at 220 nm. The concentration determined based on the calibration curve was accurate within 5%.

To determine the yield from a single macrobead, the crude β-peptide **3** mixture from a single bead was dissolved in 30 μL of DMSO. External standard was added, the solution was diluted with DMSO to a total volume of 100 μL, and 30 μL of the sample was analyzed by HPLC. The peak area ratio was measured, and the concentration was determined using the calibration curve. The amount of β-peptide **3** was calculated [concentration (mM) × volume (100 μL) = nmol of β-peptide **3**]. The percent yield was calculated based on the theoretical yield [(0.48 mmol/g loading)/(8150 macrobeads/g) = 59 nmol/macrobead]. The average yield from a single macrobead was 8 nmol (13%). This low yield was general among microwave, oil bath, and manual syntheses (data not shown). A prior screening of cleavage conditions (time: 2 or 6 h; temperature: RT or 50 °C in an incubator; and DCM concentration: 0−90% in 10% increments) identified the reported conditions that gave this yield.

Octa-β-peptide Library Synthesis on PS Macrobeads with Microwave Irradiation. Loaded PS macrobeads (62 mg, 29.8 μmol, ∼500 beads) were partitioned into five equal aliquots by weight, placed in modified SPE tubes (4 mL, Alltech), and swelled with DMF for approximately 10 min. The resin was washed (5 × DMF, 5 × CH_2Cl_2, and 5 × DMF). Deprotection solution (750 μL of 20% piperidine in DMF (v/v)) was added to the resin, and the tube was placed inside a glass 10 mL microwave reaction vessel containing ∼2 mL of DMF. A N_2 line was inserted for agitation, and the vessel was placed in the microwave reactor (CEM Discover) and irradiated (50 W maximum power, 60 °C, ramp 2 min). The sample was removed from the microwave reactor and cooled at room temperature for 10 min while the other four samples were each irradiated in turn. This ramp/cool cycle was repeated a total of 3 times per deprotection for each sample. The resin was then washed as before. Fmoc-β^3-hPhe-OH (36.2 mg, 90 μmol) was activated by adding HBTU (180 μL of 0.5 M solution in DMF), DMF (1.32 mL), HOBt (180 μL of 0.5 M solution in DMF), and iPr$_2$EtN (180 μL of 1.0 M solution in DMF). The mixture was vortexed, and 372 μL of the coupling solution was added to each aliquot of resin. The first sample was irradiated in the microwave reactor (50 W maximum power, 50 °C, ramp 2 min), removed from the reactor, and cooled for 10 min at room temperature, while the other four samples were each irradiated in turn. This ramp/cool cycle was repeated a total of 6 times per coupling step for each sample. A few beads were removed from the first sample and washed as described. The beads were suspended in ∼1 mL of DMF, and iPr$_2$EtN (30 μL of 1.0 M solution in DMF) and TNBS (30 μL of a 1% solution in DMF) were added. After 5 min, the center of the beads was stained red, indicating the presence of free amino groups and an incomplete coupling reaction. The five samples were irradiated for an additional 3 ramp/cool cycles, after which the TNBS test was negative (≤5% of amino groups are unreacted). Washing and deprotection were performed as described. Then, 18 μmol of Fmoc-β^3hVal-OH (6.4 mg), Fmoc-βhGly-OH (5.6 mg), Fmoc-β^3hAla-OH (5.9 mg), Fmoc-β^3hLeu-OH (6.6 mg), and Fmoc-(*S*,*S*)-ACHC-OH (6.6 mg) were each activated with HBTU (36 μL of 0.5 M solution in DMF), DMF (264 μL), HOBt (36 μL of 0.5 M solution in DMF), and iPr$_2$EtN (36 μL of 1.0 M solution in DMF). After vortexing, each coupling mixture was added to a different aliquot of resin, and 6 cycles of microwave ramp/cool cycles were performed for each sample. A TNBS test of each aliquot of resin at the end of the reaction was negative. After washing and deprotection, the resin was combined, suspended in DMF, and thoroughly mixed. The resin was partitioned into four approximately equal aliquots. Then, 22.5 μmol of Fmoc-β^3hOrn(Boc)-OH (10.6 mg), Fmoc-β^3hGlu(*t*Bu)-OH (9.9 mg), Fmoc-β^3hSer(*t*Bu)-OH (9.0 mg), and Fmoc-(*S*,*S*)-APiC(Boc)-OH (10.5 mg) was activated with HBTU (45 μL of 0.5 M solution in DMF), DMF (330 μL), HOBt (45 μL of 0.5 M solution in DMF), and iPr$_2$EtN (45 μL of 1.0 M solution in DMF). After vortexing, each coupling mixture was added to a different aliquot of resin, and 6 cycles of microwave ramp/cool cycles were performed for each sample. Fmoc-(*S*,*S*)-APiC(Boc)-OH was double-coupled to its respective resin aliquot. A TNBS test of each aliquot of resin at the end of the coupling reaction was negative. The resin was combined, suspended in DMF, and thoroughly mixed. The resin was partitioned into five approximately equal aliquots. Following washing and deprotection, Fmoc-β^3hTrp(Boc)-OH (48.7 mg) was coupled using the same procedure as described for Fmoc-β^3-hPhe-OH. A TNBS test of each aliquot of resin at the end of the reaction was negative. Following deprotection and washing, Fmoc-β^3hVal-OH, Fmoc-βhGly-OH, Fmoc-β^3hAla-OH, and Fmoc-β^3hLeu-OH were coupled as before. Fmoc-(*S*,*S*)-ACHC-OH was coupled to the fifth aliquot in 0.8 M LiCl in NMP. Since this was the sixth residue from the C-terminus, each residue was double-coupled in its respective solvent. A TNBS test of each aliquot of resin at the end of the second coupling reaction was negative. Washing and double-deprotection was performed, followed by coupling of Fmoc-β^3hGlu(*t*Bu)-OH (39.6 mg). Deprotection, washing, and coupling of Fmoc-β^3hLeu-OH (33.1 mg) was followed by a final deprotection. The resin was combined, washed (5 × DMF, 5 × CH_2Cl_2, 5 × DMF, 5 × CH_2Cl_2, and 5 × MeOH), and dried under a stream of N_2 until it was free-flowing. The macrobeads were arrayed (1 bead per well) into five polypropylene V-bottom 96-well plates (Greiner) using tweezers. The β-peptides were cleaved from the solid support with simultaneous side chain deprotection (110 μL, 50:50:5:5 TFA/CH_2Cl_2/TES/water, 2 h, RT, with orbital shaking; the plate was sealed with a polyolefin mat cover from Fisher Scientific). At the end of the reaction, the covered plate was centrifuged (1250 rpm, 1 min) to remove resin and cleavage solution from the cover. The cover was then removed, and the cleavage solution was concentrated by rotary evaporation (RT, 1 h, SpeedVac, Thermo Savant). The crude β-peptide mixtures were dissolved in 3 μL of DMSO; 2 μL of this stock solution was used for the ELISA screening, while 1 μL was reserved for compound identification. The crude β-peptide mixtures from 50 beads were dissolved in 30 μL of DMSO for HPLC analysis (Shimadzu); 20 μL was injected on a C_4-silica reverse-phase analytical column (5 μm, 4 mm × 250 mm, Vydac) and eluted with a gradient of acetonitrile in water (10−60%, 50 min, 0.1% TFA in each) at a flow rate of 1 mL/min. The product purity was determined as peak area percent by integration of the UV absorbance at 220 nm. Integration was performed over the 15−50 min time interval to exclude the large absorbance of DMSO that elutes from 5 to 15 min. The lower threshold of integration was set to exclude minor peaks whose areas were <1% of the peak area of the major species. The major peak in each HPLC run was collected, and β-peptide masses were measured by MALDI-TOF-MS (Bruker Reflex II, α-cyano-4-hydroxycinnamic acid matrix).

HPLC ESI-MS/MS Analysis of β-Peptides from Library. The μLC-MS/MS system consisted of an HPLC connected to an ESI ion trap mass spectrometer (Surveyor HPLC and LCQ deca XPplus, ThermoFinnigan, San Jose, CA). A fritless 100 × 365 μm fused-silica capillary microcolumn was prepared by pulling the tip of the capillary to approximately 2 μm with a P-2000 laser puller (Sutter Instruments Co.) and packing with 10 cm of C_{18}-silica beads (5 μm diameter, Western Analytical Products, Inc, Murrieta, CA). The capillary column was connected to the HPLC through a PEEK microcross with a platinum wire inserted into the flow-through to supply a spray voltage of 1.8 kV. The remaining 10 μL of β-peptide DMSO stock solution from 10 of the randomly selected macrobeads was diluted 1:1 with 95% H_2O, 0.1% formic acid/5% acetonitrile, 0.1% formic acid (95% buffer A, 5% buffer B). Alternatively, the remaining 1 μL of β-peptide DMSO stock solution from the ELISA screening hits was diluted with 20 μL of 95% buffer A, 5% buffer B. A total of 10 μL of either β-peptide solution was loaded onto the fused-silica capillary microcolumn at a flow-rate of 1 μL/min (95% buffer A, 5% buffer B) for 20 min. A gradient from 5% buffer B to 80% buffer B was run over 74 min at a

flow-rate of 300 nL/min to elute the mixture. The ion trap mass spectrometer was set to run in biggest 3 mode, which consists of a full-mass scan between 400 and 2000 *m/z*, followed by an MS/MS scan of each of the three highest-intensity parent ions with a normalized collision energy of 45%.

ELISA Procedure. A GST-MDM2 fusion protein containing full-length human MDM2 and His_6-tagged human p53 was expressed in *Escherichia coli* and affinity purified by binding to glutathione-agarose and Ni^{2+}-NTA beads under nondenaturing conditions using standard protocols. ELISA plates were incubated with 2.5 μg/mL His6-p53 in phosphate buffered saline (PBS) for 16 h. After washing with PBS + 0.1% Tween 20 (PBST), the plates were blocked with PBS + 5% nonfat dry milk + 0.1% Tween 20 (PBSMT) for 0.5 h. Compounds were dissolved in DMSO. GST-MDM2 (5 μg/mL) was mixed with test compounds in PBSMT + 10% glycerol + 10 mM DTT and added to the wells. The plates were washed with PBST after incubating for 1 h at room temperature, then incubated with MDM2-specific monoclonal antibody 5B10 hybridoma supernatant diluted 1:10 in PBSMT for 1 h, followed by washing and incubation with HRP-rabbit-anti-mouse Ig antibody for 1 h. The 5B10 antibody recognizes a C-terminal epitope on MDM2,[43] thus ensuring that the assay detects full-length MDM2 binding to p53. The plates were developed by incubation with TMB peroxidase substrate (KPL) and measured by absorbance at 450 nm. β-Peptide hits from the initial library screening were individually resynthesized using microwave irradiation,[10] purified by preparative RP-HPLC, and retested for validation (see Supporting Information).

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Supporting Information Available: Complete ref 27d, characterization of β-peptides **1**−**4** and combinatorial library members (including sequencing), calibration curve for yield determination, additional ELISA data, and molecular modeling details. This material is available free of charge via the Internet at http://pubs.acs.org.

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