

Articles

Bicyclic Homodetic Peptide Libraries: Comparison of Synthetic Strategies for Their Solid-Phase Synthesis

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Preliminary studies and synthesis development for the preparation of a bicyclic homodetic peptide library have been carried out using orthogonal protection schemes. The best results have been obtained using two Fmoc/*t*Bu-based strategies, in which the first cycle is carried out in the solid phase through side chain functional groups previously protected with Aloc/Al groups. The second cycle is performed either in the solid phase, which requires side chain anchoring of a trifunctional amino acid and Dmb protection for the C-terminus carboxyl group, or in solution, which requires the use of highly labile resins, such as the 2-chlorotrityl (Barlos) resin. Only when the cycles are formed in a ziplike manner, that is, first the small cycle and then the larger ring, is the desired final product obtained.

Introduction

Since the late 1940s, when the antibiotic gramicidin S was found to be a cyclic peptide,¹ interest in this kind of peptide system has increased dramatically. Indeed, many pharmacologically important peptides are constrained by cyclization.^{2,3} Cyclic structures often exhibit improved metabolic stabilities, increased potencies and bioavailabilities, and better receptor selectivities.⁴ Structurally, some of these examples are *homodetic*, that is, they only have peptide (lactam) linkages connecting the constituent amino acid residues, whereas others are *heterodetic*, that is, they include other functions, such as disulfide, ester (lactone), ether, or thioether bridges that contribute to the ring(s). From a synthetic point of view cyclic peptides have attracted the attention of chemists because of the extra level of synthetic complexity required for their formation.⁵

Cyclic peptides have traditionally been prepared entirely in solution or, alternatively, by solid-phase chain assembly of the linear sequence followed by release from the support and cyclization in solution.⁶ However, within the past two decades, numerous examples have been reported in which cyclizations could be performed while peptides remain anchored to polymeric supports.⁷ This approach can take advantage of the pseudodilution phenomenon associated with

the solid-phase mode, a situation that favors intramolecular over intermolecular reactions.⁸

Homodetic cyclic peptides can be classified according to the cyclization topology,⁹ and five subclasses can be described: (i) *head-to-tail*, in which the ring is formed by cyclization of the two functional termini, the amino and the carboxyl groups; (ii) *side chain-to-side chain*, in which the cyclization connects side chain functional groups; (iii) *side chain-to-end*, in which an amino or carboxylic side chain is linked to either an N- or C-terminus; (iv) *backbone-to-backbone*, in which amide nitrogens of the peptide backbone are connected through a bridge consisting of alkyl groups and an amide bond;¹⁰ and (v) *branched*, in which two lactams connect two peptide chains.

A greater degree of rigidity can be achieved by the combination of two types of cyclic topologies. Thus, peptides containing two extra amide bonds, one between the amino and the carboxyl terminus and the second between two side chains, present a bicyclic structure. The synthesis of these peptides is highly complex, because an extra level of orthogonality is required to drive regioselectively the amide bond formation.¹¹ The work described here concerns an in-depth study of the synthesis of bicyclic homodetic peptides using the solid-phase mode. The possibility of carrying out both cyclizations on the solid phase was investigated along with the order of formation of the cyclic species and the suitability of the protecting groups used for the functions involved in the formation of the rings.¹²

MEN 10627 (Figure 1), the bicyclic hexapeptide {*cyclo*[(Met-Asp-Trp-Phe-Dpr-Leu)*cyclo*(2 β –5 β)]} that has been

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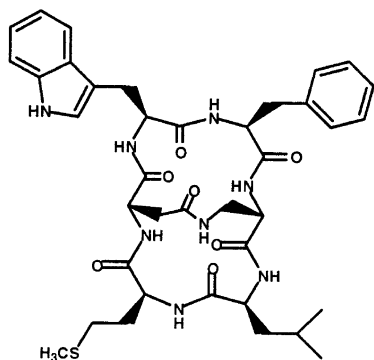


Figure 1. MEN 10627, *cyclo[(Met-Asp-Trp-Phe-Dpr-Leu)cyclo(2 β -5 β)]*.

reported to be a potent and selective peptide-based tachykinin NK2 receptor antagonist,¹³ was chosen as a model for the present study. MEN 10627 is formed by two fused 14-membered rings, and each of these contains two consecutive α -amino acids (Trp³-Phe⁴ and Leu⁶-Met¹) followed by two β -amino acids (Asp² and Dpr⁵). Both rings have a β -turn conformation, with Trp and Leu at $i + 1$ and Phe and Met at $i + 2$ positions, respectively; the Trp-Phe turn is of type I, and Leu-Met, of type II.¹⁴

The ultimate aim of our project will be to use this kind of compound in a combinatorial program as scaffolds for the display of functional groups that are known to be involved in interactions with the corresponding receptors. Preliminary studies and synthesis development¹⁵ for the preparation of a bicyclic peptide library will be described and discussed.

Results and Discussion

Since Merrifield first described the solid-phase peptide synthesis approach,¹⁶ only two protection schemes have been widely adopted: the Boc/Bzl strategy, which depends on differences in lability in the presence of acids, and the Fmoc/*t*Bu approach, which is based on the orthogonal concept that uses piperidine and TFA, respectively, to remove the two kinds of protecting groups.¹⁷ Although the first approach has given excellent results in the synthesis of thousands of peptides, the second method is more convenient in the synthesis of complex peptides, such as bicyclic systems, in which orthogonality should be introduced.¹⁸ An additional advantage of the Fmoc/*t*Bu strategy is the avoidance of the use of strong acids, such as HF or trifluoromethanesulfonic acid, is not required. This aspect should also favor the automation and parallelization of this process for the production of libraries based on bicyclic peptides.

Considering MEN 10627 as a model, an initial study consisting of six different strategies was undertaken (Figure 2).

In all cases, the Fmoc group was used as a temporary α -amino protecting group and the Boc group was used to protect the side chain of Trp. Although it is not strictly necessary to protect the indole ring, it has been shown that the Boc group suppresses undesired alkylations during the removal of other protecting groups.^{19,20} In the six strategies represented above, the first cycle was carried out while the peptide chain remained anchored on the resin. Furthermore, in strategies 5 and 6, the second cycle was performed in solution after cleavage of the peptide from the resin, but in

strategies 1–4 the second cycle was carried out on solid phase prior to final cleavage from the resin. In these cases, the amino acid Leu was replaced by Asp, which was anchored through the β -carboxyl group to an AM-resin and, therefore, gave an Asn residue after final cleavage.²¹ In all cases, the amino acid anchored to the resin was the one in position 6 (Leu in strategies 5 and 6 or Asp in strategies 1–4) because, in a previous synthesis of MEN 10627 reported by one of us,²² it was demonstrated that macrocyclization through acylation of the amino function of Met takes place in good yields. Furthermore, in all cases, the harsher treatments with Pd⁰ and hydrazine were carried out while peptides remained on the solid phase, which greatly facilitates the workup of these reactions.

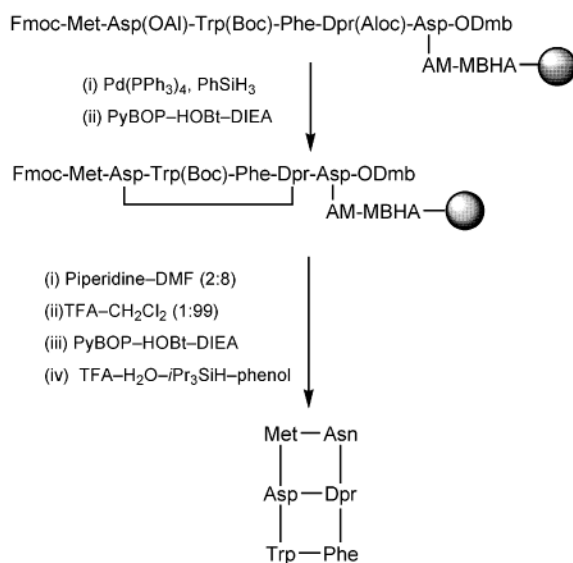
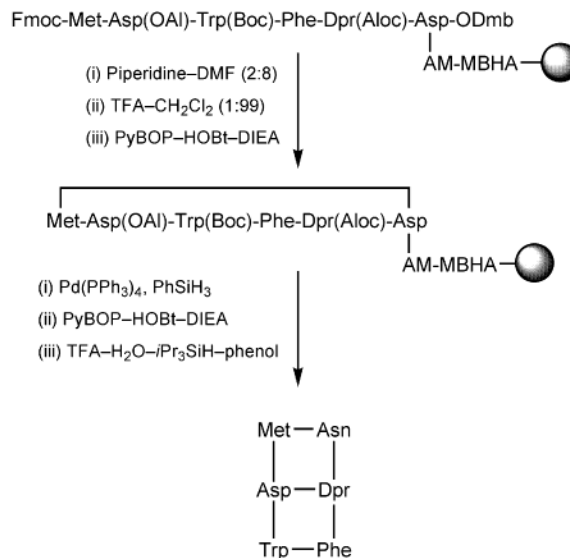
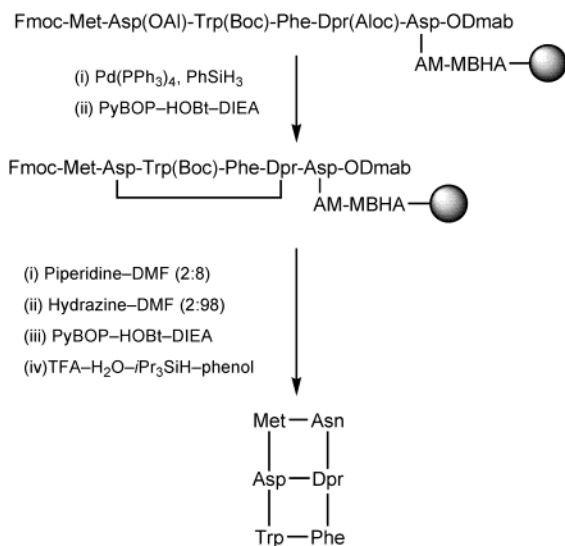
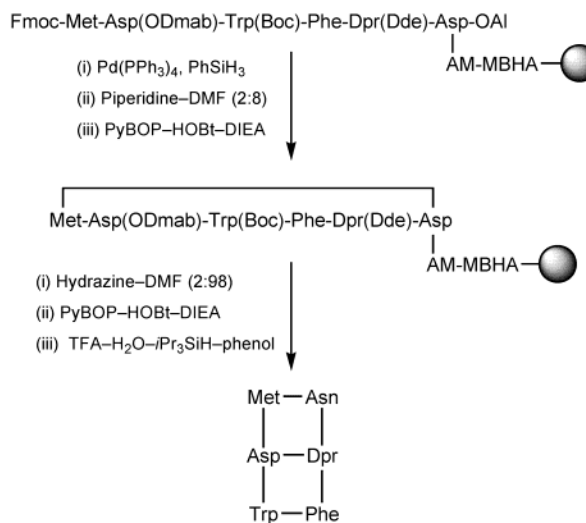
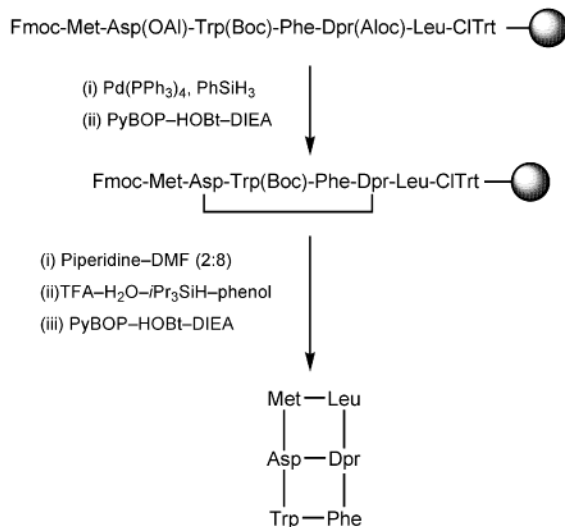
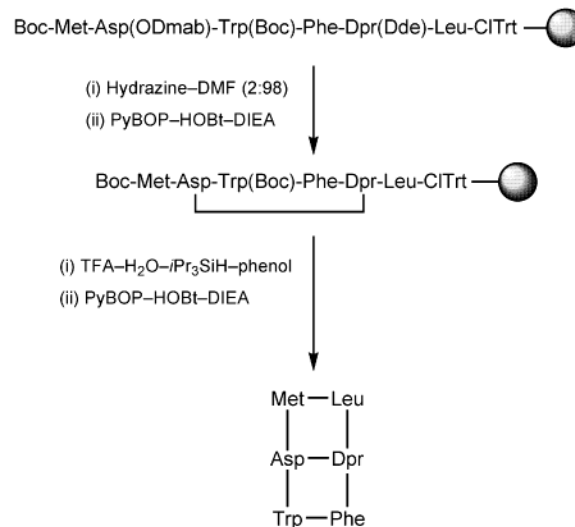
In strategies 1–4, for the side chains of Asp and Dpr and the C-terminus carboxyl group of Asp, it is necessary to use two kinds of protecting group in an orthogonal manner, or at least, they should be compatible with the Fmoc group (removed with piperidine) and the linker AM (removed by high concentrations of TFA)²³

For strategies 1 and 2, Aloc/Al were used for the amino and the carboxyl side chains of the central Asp and Dpr in addition to Dmb for the C-terminus of Asp. The first groups are stable to bases/nucleophiles and acids and are removed under practically neutral conditions through a Pd-catalyzed transfer of the allyl moiety to a nucleophile (allyl scavenger).²⁴ The choice of an appropriate allyl scavenger is crucial to establish a convenient strategy for this semipermanent protecting group, because allylamines could be formed as a side product. Although amine–borane complexes (e.g., H₃N–BH₃ and Me₂NH–BH₃) appear to be the best-suited scavengers for the allyl moiety,^{25,26} PhSiH₃, which is more user-friendly, has also been shown to be effective²⁷ and was, therefore, used in this work.

The Dmb can be removed with very low concentrations of TFA, and this does not have any effect on the anchoring of the peptide to the AM resin.²⁸ Differences between strategies 1 and 2 lie in the order in which the cycles were formed (smaller cycle first in strategy 1 versus larger cycle first in strategy 2).

In strategies 3 and 4, nucleophile-labile protecting groups were used together with the Aloc/Al groups. Dmab²⁹ was used for the C-terminus and the β -carboxyl groups of both Asp units. Dde³⁰ was employed for the side chain of the Dpr. Despite the fact that Dmab/Dde is stable to piperidine and can be removed by hydrazine, it is not orthogonal with respect to the Fmoc group, but is simply compatible with it. In this case, the Fmoc group, which is not stable to hydrazine, should be removed first. Strategy 3 is similar to strategy 1 but Dmab has replaced Dmb. Once again, differences between strategies 3 and 4 lie in the order of ring formation. In strategy 4, the position of the protecting groups has also been switched (i.e., Al for the C-terminus of Asp and Dmab/Dde for the side chain functions).

In strategies 5 and 6 (first cycle in solid phase and second in solution), the pairs Aloc/Al (strategy 5) and Dde/Dmab (strategy 6) were investigated to mask the functional groups that will be used to form the first cycle. In both cases, Barlos (2-chlorotrityl chloride) resin³¹ was used, because it allows

Strategy 1. Solid-phase/Solid-phase**Strategy 2. Solid-phase/Solid-phase****Strategy 3. Solid-phase/Solid-phase****Strategy 4. Solid-phase/Solid-phase****Strategy 5. Solid-phase/Solution****Strategy 6. Solid-phase/Solution****Figure 2.** Strategies for the synthesis of the bicyclic peptide.

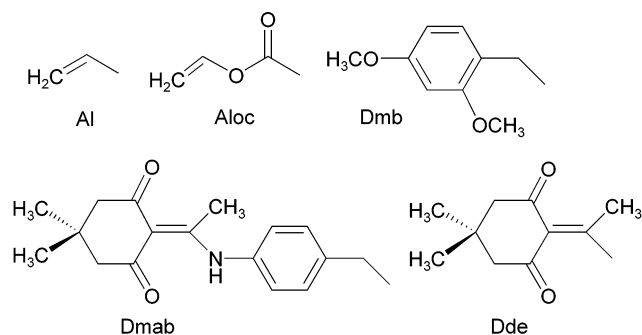


Figure 3. Structures of different protecting groups used.

cleavage of the peptide under very mild conditions and in the presence of *t*Bu-based protecting groups. In strategy 6, in which the Dde/Dmab pair was used, the Met unit was introduced in its protected form with the acid-labile Boc group because, as discussed previously, the Fmoc group is not stable to treatment with hydrazine.³²

The key steps in this approach are the two cyclization reactions. Although the best cyclization reaction conditions to form each cyclic peptide could require fine-tuning, it is a prerequisite in all combinatorial programs to reduce the number of reagents to a minimum for a common step. For this reason, it is desirable to use just one cyclization protocol in the work described here. It is widely accepted that aminium and phosphonium salts are the most powerful coupling reagents.³³ Aminium reagents should be used with care because they can react with the amino component, leading to the corresponding guanidino derivatives.³⁴ This side reaction is not important during the coupling of single protected amino acids because activation is fast and the aminium salt is rapidly consumed. However, cyclization is frequently a slow reaction, and excesses of coupling reagents are usually used. In this case, the aminium salt can react with the amino component. On the other hand, phosphonium salts that show similar reactivity to the aminium species do not promote this side reaction.^{34b} It is therefore possible, and indeed convenient, to add extra equivalents of phosphonium salts during the course of the cyclization to ensure the activation of the carboxylic acid. The main advantage of this technique is that it overcomes the fact that active species are not completely stable and during slow reactions, such as cyclization, they can be hydrolyzed.^{34b} Pyrrolidino derivatives are preferred to dimethylamino ones because the latter liberate hexamethylphosphoric triamide, a compound that has been classified as a potential human carcinogen.³⁵ The most widely used pyrrolidino phosphonium salts are derivatives of HOBt and HOAt, i.e., PyBOP³⁶ and PyAOP,³⁷ respectively. Although it has often been demonstrated that PyAOP is superior to PyBOP,³⁷ the latter was chosen for reasons of economy.³⁸

Solid-phase cyclizations were carried out with PyBOP/HOBt/DIEA (3:3:6) for 60 min and, after filtration and washing, the course of the reaction was checked by the ninhydrin test. The process was repeated until a negative test result was obtained.³⁹ Solution cyclizations were also performed at 10^{-2} M with PyBOP/HOBt/DIEA (10:10:20) for 3 days, with addition of a further quantity of PyBOP (5 equiv) after the second day. In this strategy (solid phase/solution), the excess HOBt and other coupling reagent

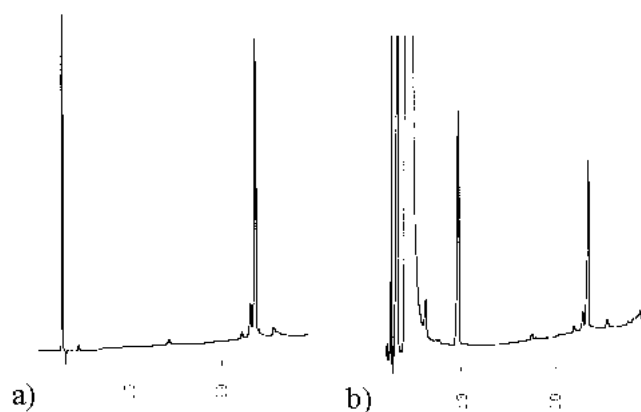


Figure 4. HPLC of crude peptides obtained by strategies 1 (a) and 5 (b). Conditions: Kromasil C₁₈ 10- μm (250 \times 4.6 mm), linear gradient from 5 to 95% acetonitrile (+0.036% TFA) in water (+0.045% TFA) in 30 min, 1 mL/min, detection at 220 nm.

derivatives can be almost completely removed by trituration with TBME and decantation.

The progress of the synthesis was checked at different stages (after elongation of the peptide sequence, formation of the first cycle, and final product) by HPLC and MALDI-TOF spectra of products obtained after cleavage with reagent B [TFA/H₂O/*i*Pr₃SiH/phenol (90:3:2:5)].⁴⁰ The main conclusions of this part of the study are: (i) in all cases when the desired peptide was obtained, it was accompanied by a major impurity corresponding to the peptide with the residue of Met in the form of Met(O);⁴¹ (ii) the undesired side reaction was completely avoided by adding TMSBr and EDT to the TFA cleavage cocktail;^{42,43} (iii) the formation of the first cycle on the resin was in all cases achieved satisfactorily; (iv) comparison of strategies 1 and 2, i.e., assessing the influence of the order of formation of the cycles, shows that only when the cycles are formed in a zip-like manner (forming the small cycle first) is the desired final product obtained; (v) strategies involving the use of the protecting groups Dmab and Dde (strategies 3, 4 and 6) clearly gave rise to poor-quality final products;^{44,45} (vi) the best two strategies (one solid phase/solid phase and one solid phase/solution) were 1 and 5, which are based on the use of Al/Alloc for Asp and Dpr, respectively, and a highly acid-labile protecting group for the remaining carboxylic group (Dmb in strategy 1 and ClTrt-resin in strategy 5); and (vii) comparison of these two latter strategies serves to compare solid phase vs solution; although it can be seen from the HPLC of the crude products (Figure 4) that the quality of both crude materials is quite similar,⁴⁶ the main advantages of strategy 1 (both cycles performed in solid phase) is that the workup is much simpler (no need to remove either side products or high-boiling solvents, such as DMF). This advantage is particularly important for library production.⁴⁷

To validate our approaches, 24 further sequences based on MEN 10627 were synthesized (12 peptides following each strategy, Figure 5a,b). Both series contain the same peptides, with the replacement of Leu (strategy 5, solid phase/solution) by Asn (strategy 1, solid phase/solid phase) being the only change. New sequences involved the presence of trifunctional



Figure 5. a. Sequences synthesized using strategy 1 (solid phase/solid phase). Asp was used as a replacement for Leu. The residue in bold denotes the one that is different from the wild-type sequence. b. Sequences synthesized using strategy 5 (solid phase/solution). The residue in bold denotes the one that is different from the wild-type sequence.

amino acids, D-amino acids, and N-alkylamino acids in positions 1, 3, and 4 (Met, Trp, and Phe in the wild-type sequence).

The progress of each synthesis was checked by HPLC after the first cyclization (for syntheses 1–12, a small aliquot of peptide resin was cleaved) and at the end of the process. HPLC analysis after the formation of the first cycle showed that in all 24 cases, the cycle had formed with excellent yields and purity (>95% for both parameters). The results in Table 1 relate the HPLC analysis of the final products that are summarized as follows: (i) Both strategies work similarly and allow the preparation of the majority of the peptides. (ii) The second cycle is best formed by strategy 1 (solid phase/solid phase), because there are only three cases (entries 2, 4, and 6) in which it is not quantitatively formed, while for strategy 5, there are seven cases (entries 16, 18–22, and 24). These results, in addition to the fact that a completely solid-phase procedure is more advantageous, makes this the strategy of choice (wherever possible) for the production of a bicyclic peptide-based library. (iii) In general, the presence

of D-amino acids does not favor the formation of the desired compound. Thus, the inclusion in position 1 (entries 2 and 14), 3 (entries 3 and 15), and 4 (entries 4 and 16) of the corresponding D-residue gave worse results in all cases when compared with the wild sequence. (iv) The desired product was obtained in all cases, except when Pro (entries 11 and 23) was inserted instead of Phe. Furthermore, when another N-alkylamino acid, such as Sar (entries 10 and 22), is placed in the Phe position, the results are also worse. On the other hand, when D-Pro (entries 12 and 24) was used instead of Pro/Phe, the desired product was obtained. These results do not support the idea that the presence of both D- and N-alkylamino acids favors peptide cyclization.² It is worth noting that in further syntheses following strategy 1, it is necessary to check the formation of the second cycle with more than one colorimetric test,⁴⁸ because although the monocyclic peptide was detected in the final crude product (entries 2, 4, and 6). Furthermore, additional colorimetric tests should also be used for the control of the first cycle,

Table 1. HPLC Purities and MS of Bicyclic Peptides

entry	sequence: X ₁ X ₂ X ₃ X ₄ X ₅ X ₆ ^a	strategy ^b	peptide content (%) ^c	monocycle in crude (%) ^c	bicycle in crude (%) ^c	cyclization time (h)		MS (M + H) ⁺ ^d	
						1st cycle	2nd cycle	calcd	found
1	Met Asp Trp Phe Dpr Asn	1	81		81	3	1	763.36	762.07
2	DMet Asp Trp Phe Dpr Asn	1	94	62	32	2	2	763.36	762.17
3	Met Asp DTrp Phe Dpr Asn	1	53		53	3	1	763.36	762.10
4	Met Asp Trp DPhe Dpr Asn	1	98	84	14	3	3	763.36	762.12
5	Cys Asp Trp Phe Dpr Asn	1	81		81	2	3	735.28	734.09
6	Met Asp Trp Tyr Dpr Asn	1	96	19	77	3	2	779.20	778.16
7	Met Asp Phe Phe Dpr Asn	1	66		66	3	2	724.30	723.47
8	Met Asp Trp Ile Dpr Asn	1	54		54	3	2	690.31	689.56
9	Met Asp Trp Ala Dpr Asn	1	83		83	3	1	648.26	647.38
10	Met Asp Trp Sar Dpr Asn	1	0			1	3	648.26	
11	Met Asp Trp Pro Dpr Asn	1	0			1	2	674.28	
12	Met Asp Trp DPro Dpr Asn	1	80		80	3	1	674.28	675.40
13	Met Asp Trp Phe Dpr Leu	5	42		42	3	72	761.36	761.52
14	DMet Asp Trp Phe Dpr Leu	5	54		54	3	72	761.36	761.54
15	Met Asp DTrp Phe Dpr Leu	5	0			3	72	761.36	-
16	Met Asp Trp DPhe Dpr Leu	5	70	12	58	3	72	761.36	761.21
17	Cys Asp Trp Phe Dpr Leu	5	34		34	3	72	733.33	733.06
18	Met Asp Trp Tyr Dpr Leu	5	68	21	47	3	72	777.36	776.99
19	Met Asp Phe Phe Dpr Leu	5	100	60	40	3	72	722.35	722.07
20	Met Asp Trp Ile Dpr Leu	5	100	21	79	3	72	727.38	727.49
21	Met Asp Trp Ala Dpr Leu	5	92	45	47	3	72	685.33	685.55
22	Met Asp Trp Sar Dpr Leu	5	50	30	20	2	72	685.33	685.25
23	Met Asp Trp Pro Dpr Leu	5	0			3	72	711.35	
24	Met Asp Trp DPro Dpr Leu	5	99	64	35	3	72	711.35	711.38

^a For strategy 1 (entries 1–12), Asn was used as replacement of Leu. The residue in bold denotes the one that is different from the wild sequence. ^b Strategy 1 = both cycles in solid phase; strategy 5 = first cycle in solid phase and second cycle in solution. ^c Yields have been calculated by HPLC without considering peaks corresponding to nonpeptidic material or, in strategy 2, those corresponding to coupling reagent derivatives. ^d PerSeptive Biosystems Voyager DE-RP, N₂ laser (337 nm) reflectron mode and delayed extraction, 25 kV voltage, using 2,5-dihydroxybenzoic acid (DHB) as matrix.

because in some cases (entries 7, 8, 13, and 17), the purity of the target peptide was low, despite the fact that the monocycle was not detected in the crude material.

Conclusions

Bicyclic homodetic peptide libraries can be synthesized using two Fmoc/*t*Bu-based strategies in which the first cycle is carried out in the solid phase through side chain functional groups previously protected with Alloc/Al groups, and the second is performed either in solid phase (strategy 1), which requires side chain anchoring of a trifunctional amino acid and Dmb protection for the C-terminus carboxyl group, or in solution (strategy 5), which requires the use of highly labile resins, such as the Barlos resin. In the second case, a concentration of 10⁻² M was found for to be optimal for the solution cyclization because it represents a balance between precluding the formation of intermolecular linkages and avoiding the use of large amounts of DMF that need to be removed in the subsequent step. Furthermore, only when cycles are formed in a ziplike manner, that is, first the small ring formation and then the larger ring, is the desired final product obtained. Finally, the presence of both D- and N-alkylamino acids does not favor peptide cyclization.

Materials & General Methods

Materials and Equipment. Protected amino acids were obtained from Luxembourg Industries (Tel-Aviv, Israel), Neosystem (Strasbourg, France), Calbiochem-Novabiochem AG (Läufelfingen, Switzerland), and Bachem AG (Bubendorf, Switzerland). PyBOP, Fmoc-AM handle, and solid

supports were supplied by Calbiochem-Novabiochem AG. DIPCDI was obtained from Fluka Chemika (Buchs, Switzerland), and HOBt, from Albatross Chem Inc. (Montreal, Canada). Solvents for peptide synthesis and RP-HPLC were obtained from Scharlau (Barcelona, Spain). Trifluoroacetic acid was supplied by KaliChemie (Bad Wimpfen, Germany). Other chemicals used were obtained from Aldrich (Milwaukee, WI) and were of the highest purity commercially available. All commercial reagents and solvents were used as received, with the exception of DMF and DCM, which were bubbled with nitrogen to remove volatile contaminants (DMF) and stored over activated 4A molecular sieves (Merck, Darmstadt, Germany) (DMF), passed through a short column of Al₂O₃ (DCM for peptide synthesis), or stored over CaCl₂ (DCM for Pd chemistry). HPLC was performed using a Shimadzu (Kyoto, Japan) chromatography system with a reversed-phase Kromasil C₁₈ (250 × 4 mm) 10-μm column with UV detection at 220 nm. Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (Applied Biosystems, Framingham).

Peptide resin samples were hydrolyzed in 12 N aqueous HCl/propionic acid (1:1, v/v) at 155 °C for 1–3 h. Subsequent amino acid analyses were performed on a Beckman System 6300 autoanalyzer (Fullerton, CA).

General Procedures. Solid-phase peptide elongation (first six synthesis) and other solid-phase manipulations were carried out in polypropylene syringes fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Washings between deprotection, coupling, and subsequent deprotection steps were carried out with

DMF (5 × 0.5 min) and DCM (5 × 0.5 min) using 10 mL of solvent/g of resin each time.

Syntheses using Fmoc-AM resin (strategies 1–4) were carried out with 50 μ mol (98 mg) of Fmoc-AM resin (0.51 mmol/g). In the cases in which the Barlos resin was used (strategies 5 and 6), syntheses started with 100 mg of resin (nominal loading, 1.5 mmol/g), but after the partial incorporation of the first Fmoc-amino, the loading was 0.45–0.50 mmol/g.⁴⁹

Solid-phase peptide elongation (24 synthesis) was carried out in a simultaneous multiple peptide synthesizer (AM₄₂₂, Abimed Analyzentechnik, Langenfeld, Germany) on a 50- μ mol scale, with a 20-min deprotection step with piperidine/DMF (2:8, v/v) and a 45-min basic coupling time with Fmoc-amino acid/TBTU/NMM (4:4:8) without preactivation.

Manual Removal of Protecting Groups. Fmoc: piperidine/DMF (2:8, v/v) (2 × 10 min); Alloc/Al: Pd(PPh₃)₄ (0.1 equiv), PhSiH₃ (10 equiv) in anhydrous DCM (3 × 15 min) under an Ar atmosphere;¹³ Dmab/Dde: hydrazine monohydrate/DMF (2:98) (3 × 3 min); Dmb: TFA/DCM (1:99, v/v) (1 × 30 min).

Coupling. Protected amino acid (3 equiv) in DMF (1–3 mL/g resin) and DIPCDI (3 equiv) were sequentially added to the resin and allowed to react with intermittent manual stirring for 1 h (16 h for the incorporation of the Fmoc-AM handle onto the *p*-MBHA resin). The solvent was removed by filtration, the resin was washed as indicated above, and the extent of the coupling was checked by the ninhydrin test.

Anchoring of the first amino acid to the Barlos resin was carried out with the Fmoc-amino acid (0.5 equiv) and DIEA (5 equiv) in DCM, and the slurry was stirred for 1 h. The unreacted reactive Cl groups were capped by addition of MeOH (1 mL/g resin), and after removal of the Fmoc group, the loading was calculated by amino acid analysis of the acid hydrolyzed.

Solid-Phase Cyclization. PyBOP (3 equiv) and HOBt (3 equiv) dissolved in DMF (1–3 mL/g resin) were sequentially added to the resin, followed by DIEA (6 equiv). The mixture was allowed to react for 60 min with stirring. The solvent was removed by filtration, the resin was washed, and the cyclization was checked by the ninhydrin test. The process was repeated until a negative test was observed.

Cleavage of unprotected peptides from the resin was performed with TFA/H₂O/*i*Pr₃SiH/phenol (90:3:2:5, v/v) for 2 h (10 mL/g resin). Fifteen minutes before the end of this period, TMSBr and EDT were added (to give a final solution of 0.1 M TMSBr and 0.2 M EDT). Peptides were precipitated by the addition of cold TBME, the solution was decanted, and the solid was triturated with cold TBME, which was again decanted. This process was repeated twice.

Cleavage of partially protected peptides from the resin was carried out with TFA/DCM (2:98, v/v) (3 × 3 min) (10 mL/g resin). The filtrates were collected over pyridine/DCM (1:99) (50 mL/g resin), and the combined solutions were evaporated to dryness under reduced pressure and lyophilized.

Solution cyclizations were performed by dissolving the partially protected peptide in DMF and adding sequentially PyBOP (10 equiv) and HOBt (10 equiv), both dissolved in

DMF, and DIEA (20 equiv) (10 mM final concentration). Although cyclizations were allowed to proceed for 3 days, after the second day, an additional quantity of PyBOP (5 equiv) was added. Finally, HOAc was added to quench the reaction, and the DMF was removed under reduced pressure.

Removal in solution of the side chain protecting group (bicyclic peptide) was performed in a way similar to the solid-phase method, but using TFA/H₂O/*i*Pr₃SiH/phenol (90:3:2:5, v/v) as the initial cleavage cocktail. The successive treatments with TBME removed the large excess of PyBOP and HOBt used in the cyclization step.

Abbreviations. Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC–IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, 247, 977–983. The following additional abbreviations are used: Al, allyl; Alloc, allyloxycarbonyl; AM, *p*-(*R,S*)-*a*-{1-[(9-fluorenyl)methoxyformamido]-2,4-dimethoxybenzyl}phenoxyacetic acid; Boc, *tert*-butoxycarbonyl; *t*Bu, *tert*-butyl; Barlos resin, 2-chlorotrityl chloride resin; DCM, dichloromethane; Dde, 1-(4, 4'-dimethyl-2,6-dioxocyclohexylidene)ethyl; DIEA, *N,N*-diisopropylethylamine; DIPCDI, *N,N'*-diisopropylcarbodiimide; Dmb, dimethoxybenzyl; Dmab, 4-{*N*-[(4, 4'-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyl; DMF, *N,N*-dimethylformamide; Dpr, 1,2-*L*-diaminopropionic acid; EDT, 1,2-ethanedithiol; Fmoc, 9-fluorenylmethoxycarbonyl; HOAc, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; *p*-MBHA, *p*-methylbenzhydramine; MeOH, methanol; NMA, *N*-methylmercaptoacetamide; NMM, *N*-methylmorpholine; PyAOP, (7-azabenzotriazol-1-yloxy)-tris(pyrrolidino)phosphonium hexafluorophosphate; PyBOP, benzotriazol-1-yl-oxytris(pyrrolidino)phosphonium hexafluorophosphate; reagent B, TFA/H₂O/*i*Pr₃SiH/phenol (90:3:2:5); TBME, *tert*-butylmethyl ether; TFA, trifluoroacetic acid; TMSBr, trimethylsilyl bromide. Amino acid symbols denote the *L* configuration unless stated otherwise. All solvent ratios are volume/volume unless stated otherwise.

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- (44) Once this part of the work had been completed, several reports appeared in the literature that could explain these results. For example, Jung and co-workers showed that Dde is not completely stable to conditions used to remove the Fmoc group, and this small loss of Dde can compromise the purity of peptides. Furthermore, Dde has also been reported to undergo intramolecular *N*-to-*N'* migration, resulting in the scrambling of the group within the peptide chain (Augustyns, K.; Kraas, W.; Jung, G. *J. Pept. Res.* **1998**, *51*, 127–33). To avoid both side reactions, the 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl (*iv*Dde) group has been reported (Chabra, S. R.; Hothi, B.; Evans, D. J.; White, P. D.; Bycroft, B. W.; Chan, W. C. *Tetrahedron Lett.* **1998**, *39*, 1603–1606). The peptide group of the ABRF has also shown that the Dmab ester is not totally stable to piperidine and affords a mixture of the piperidine and the *p*-benzylamide (Medzihradsky, K. F.; Ambulos, N. P.; Khatri, A.; Osapay, G.; Remmer, H. A.; Somogyi, A.; Kates, S. A. *Lett. Pept. Sci.* **2001**, *8*, 1–12). A third side reaction associated with the joint use of Dde- and Al-based protecting groups implies the reduction of the double bond of the Al moiety during the hydrazinolysis, making the resulting propylcarbamate stable to Pd⁰ (Rohwedder, B.; Mutti, Y.; Dumy, P.; Mutter, M. *Tetrahedron Lett.* **1998**, *39*, 1175–1178). This potential side reaction does not apply to our work, because we removed the Alloc/Al group first and then the Dde/Dmab.

- (45) Resins after treatment with hydrazine changed completely in terms of their physical properties. This change was characterized by materials that appeared “wet” and had shrunk.
- (46) Peaks that appear before 10 min for strategy 5 (Figure 5b) correspond to PyBOP and HOBt derivatives.
- (47) For strategy 5, it was necessary to optimize the step involving the formation of the second cycle in solution. After carrying out several trials at different concentrations (10^{-1} – 10^{-5} M), it was found that 10^{-2} M was the highest concentration that favors the removal of DMF and avoids the formation of a significant amount of polymers.
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