The Development and Application of a Novel Safety-Catch Linker for BOC-Based Assembly of Libraries of Cyclic Peptides[||,1]

Gregory T. Bourne,[†,‡] Simon W. Golding,[†,‡] Ross P. McGeary,[†] Wim D. F. Meutermans,[†] Alun Jones,[†] Garland R. Marshall,[§] Paul F. Alewood,[†] and Mark L. Smythe*[,†]

Centre for Drug Design and Development, Institute for Molecular Bioscience, The University of Queensland, St. Lucia, 4072, Brisbane, Australia, and Department of Molecular Biology and Pharmacology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, Missouri 63110

m.smythe@imb.uq.edu.au

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Cyclic peptides are appealing targets in the drug-discovery process. Unfortunately, there currently exist no robust solid-phase strategies that allow the synthesis of large arrays of discrete cyclic peptides. Existing strategies are complicated, when synthesizing large libraries, by the extensive workup that is required to extract the cyclic product from the deprotection/cleavage mixture. To overcome this, we have developed a new safety-catch linker. The safety-catch concept described here involves the use of a protected catechol derivative in which one of the hydroxyls is masked with a benzyl group during peptide synthesis, thus making the linker deactivated to aminolysis. This masked derivative of the linker allows BOC solid-phase peptide assembly of the linear precursor. Prior to cyclization, the linker is activated and the linear peptide deprotected using conditions commonly employed (TFMSA), resulting in deprotected peptide attached to the activated form of the linker. Scavengers and deprotection adducts are removed by simple washing and filtration. Upon neutralization of the N-terminal amine, cyclization with concomitant cleavage from the resin yields the cyclic peptide in DMF solution. Workup is simple solvent removal. To exemplify this strategy, several cyclic peptides were synthesized targeted toward the somatostatin and integrin receptors. From this initial study and to show the strength of this method, we were able to synthesize a cyclic-peptide library containing over 400 members. This linker technology provides a new solid-phase avenue to access large arrays of cyclic peptides.

Introduction

Cyclic peptides are appealing targets due to the conformational restriction imposed by cyclization.[2,3] As such, they possess greater stability against enzymatic degradation and greater absorption potential and may possess higher affinity than their linear precursors due to preorganization. Some cyclic peptides are drugs in their own right; examples include octreotide[4] and cyclosporin A.[5] It is, therefore, not surprising that cyclic peptides have been primary targets for combinatorial library development.[6−12] In recent years, we have been interested in establishing solid-phase chemistries that allow the synthesis of libraries of discrete cyclic peptides (head-to-tail) in a practical and versatile manner.[13−17] The backbone

* To whom correspondence should be addressed: Tel: (+61) 7 33651271. Fax: (+61) 7 3365 1990.

† The University of Queensland.

‡ Authors contributed equally to this work.

§ Washington University School of Medicine.

|| Abbreviations used as follows: 3-Amb, 3-aminobenzoic Acid, BOC, *tert*-butoxycarbonyl; BOP, benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate; Bz, benzyl; DCC, 1,3 dicyclohexylcarbodiimide; DIC, 1,3 diisopropylcarbodiimide; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; ES-MS, electron-spray mass spectrometry; EtOAc, ethyl acetate, FMOC, fluorenylmethyloxycarbonyl; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HF, anhydrous hydrogen fluoride; HOAc, acetic acid; HPLC, high-performance liquid chromatography; MeOH, methanol; NMR, nuclear magnetic resonance; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; TFMSA, trifluroromethanesulfonic acid; THF, tetrahydrofuran.

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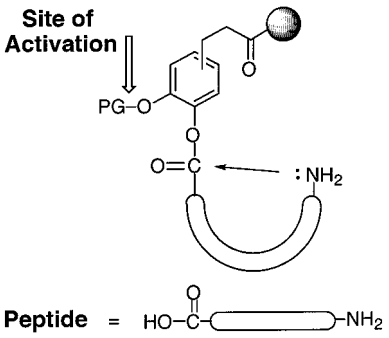
linker we have previously developed[14−16] fulfills the essential requirements of versatility but suffers from practical complications in workup. For example, after cyclization on the resin, acid-induced deprotection of side chains and cleavage from the backbone linker yields cyclic products that need to be separated from the reaction mixture. This solution-phase purification requirement adds another level of complexity to library development and restricts the use of this linker to the assembly of small libraries of cyclic peptides. In that respect, it would be useful if the deprotection and resin-cleavage steps were separated. In particular, a cleavage-by-cyclization approach where the N-terminal amine displaces the C-terminal carboxy linker after side-chain deprotection is very attractive. Indeed, such linkers would generate "clean" cyclic peptide solutions, i.e., without scavengers or acids required for resin cleavage, that are directly applicable in high-throughput screening. This would have significant impact on the throughput of library synthesis. Design of a suitable cleavage-by-cyclization linker demands that the linker be stable during assembly of the linear precursors, but should, at the same time, enable on-resin acid-induced deprotection of the side-chain functionalities, and after suitable linker activation, allow nucleophilic displacement by the N-terminal amine.

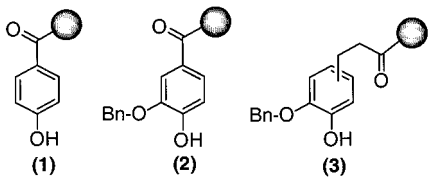
The existing linkers that enable cleavage-by-cyclization approach fall into two classes, the active-ester linkers[18,19] and the safety-catch linkers.[20−40] The most commonly used active-ester linker is Kaisers' oxime-resin[18] that is strong-acid labile and, hence, does not fit the requirements. Other active-ester linkers that have been described, such as the thioester linker,[19] are stable to strong acids; however, the nature of these linkers makes them susceptible to nucleophiles present in the reaction mixture, and hence, low yields of cyclic peptides are sometimes observed.

Scheme 1. Structure of the Safety Catch Linker[a]



[a] The arrow shows site of activation (via strong acid or HF deprotection of a suitable protecting group).

Scheme 2. Subset of Linkers Investigated



Safety-catch linkers are masked active-ester linkers[20−40] that are stable but can be chemically modified (unmasked) to render them susceptible to cleavage. Although conceptually very attractive, the chemical requirements are high and only a few examples have been reported, such as the acylsulfonamide linker,[20−23] known as Kenners safety-catch linker and Marshall's sulfone linker.[24,25] Both utilize additional chemical reagents to activate the linker (by alkylation or oxidation respectively) that, given the diverse functionalities in linear peptides, may cause side reactions and decrease the purity of the product.

The present paper reports on the development of a "new" safety-catch linker with the general structure of Scheme 1 and associated chemistries for the synthesis of head-to-tail cyclic peptides. We further describe the effective assembly of cyclic-peptide libraries using automated parallel synthesis. This linker technology provides a new solid-phase avenue to access large arrays of cyclic peptides.

Results and Discussion

Design of the Safety-Catch Linker. In our search for a suitable linker for synthesis of large arrays of cyclic peptides, we initially investigated 4-carboxyphenyl esters **1** (Scheme 2) but found that lability during assembly led to a significant amount of deletion products and impurities in the cleavage mixtures. These difficulties forced us to seek a linker that was more stable during assembly, yet labile enough to allow peptide cyclizations. This intrinsic complication led us to alter the approach and search for a suitable safety-catch linker.

In search for a new safety catch linker design that satisfies our requirements in terms of stability and noncompromising activation, we chose the *O*-hydroxyphenyl esters **2** (Scheme 2).[41−46] Ciuffarin[41,42] and Lois[42] reported that reactivity of *O*-alkoxyphenyl ester toward amines in their alkylated form are approximately 3 orders of magnitude less reactive then in their un-

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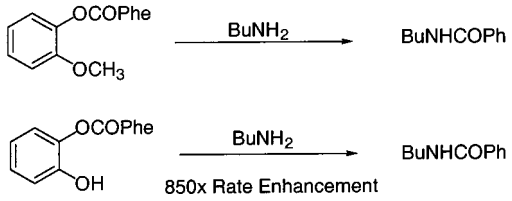
Scheme 3. Rate of Aminolysis of Ortho-Substituted Phenyl Benzoates[41]

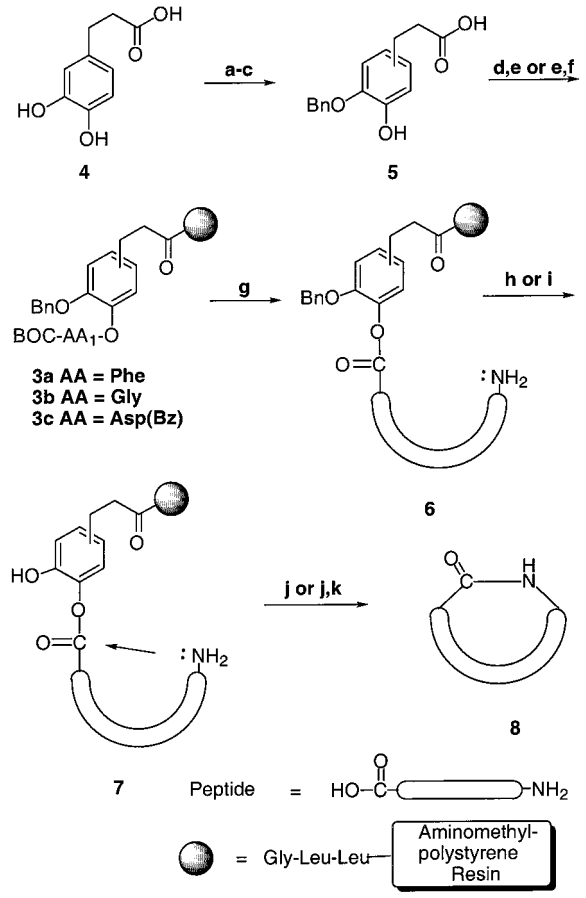


alkylated form (Scheme 3). Moreover, it was also reported that aminolysis of the *O*-hydroxyphenyl ester of protected amino acids or peptides occurs without racemization.[43,44] On the basis of these findings, Jones[43,44] and Young[43] applied the *O*-benzyloxyphenyl esters as masked active esters in solution-phase chemistry. Activation of the ester was established by removing the *O*-benzyl group (HBr/AcOH or hydrogenation), and the resulting "activated" ester was reacted in a solution-phase oligomerization or cyclization reaction. We decided to explore this concept on resin. We initially evaluated linker **2** but, again, found it to be too reactive during peptide assembly possibly due to conjugation of the carbonyl group and the aromatic ring. We postulated that to decrease the activity of the ester during peptide assembly we needed to insert an alkyl chain between these functional groups. This resulted in the design and synthesis of the safety-catch linker **3** (Scheme 2).

Synthesis of the Safety-Catch Linker. Synthesis of the linker **3** was easily accomplished in a three-step process by initially forming the methyl ester[47] followed by benzylation of one of the alcohol groups with benzyl bromide and K_2CO_3 (nonregiospecific alkylation). This was followed by hydrolysis of the resultant methyl ester with $LiOH \cdot H_2O$ to give compound **5** as a mixture of two regioisomers (Scheme 4). Since both regioisomers co-eluted by analytical and preparative HPLC, we found isolation to be difficult, in our hands. However, as we expected both isomers to contain the same chemical properties in terms of stability and reactivity toward head-to-tail cyclization, we evaluated all further chemistries using the regioisomer mixture of linkers. We therefore did not determine the aminolysis rates of the individual isomers.

Resin attachment was achieved on the aminomethyl polystyrene resin (substitution value = 0.26 mmol/g). Low loading was used to circumvent possible oligomerization when cyclizing the fully synthesized linear peptide. Attachment of the safety catch linker proceeded via two different pathways. We either attached the first amino acid residue to the linker via the unsymmetric anhydride[48] in solution followed by appendage to resin or we attached the linker to the resin followed by on resin O-acylation DIC/DMAP.[49] Solution O-acylation proceeded in 80−90% yield, while it was quantitative when performed on aminomethylated resin (containing the tripeptide spacer group Gly-Leu-Leu).[14,50]

Scheme 4. Synthesis of the Safety Catch Linker[a]



[a] Reagents and conditions: (a) MeOH, H_2SO_4, Δ, 16 h; (b) 1 equiv of BnBr, K_2CO_3, $(n\text{-Bu})_4NI$, DMF, 16 h; (c) LiOH, THF/H_2O 1:1, 3 h, rt; (d) BOC-AA-OH, EtOCOCl, DIEA, −8 °C; (e) H-Gly-Leu-Leu-aminomethyl polystyrene resin, HBTU, DIEA; (f) BOC-Asp(OBz)-OH, DIC, DMAP, rt, 2 h; (g) SPPS; (h) HF/*p*-cresol, 9:1, 1 h, −5 °C; (i) HBr/TFA, *p*-cresol, 1 h, rt; (j) 2% DIEA in DMF, 16 h, rt; (k) 20% piperidine/DMF, 2 h, rt.

This strategy was examined on an array of model peptides targeted at the somatostatin[51−53] and integrin receptors[54−56] (see Table 1) as outlined in Scheme 4. The linear peptides **6** (Scheme 4) were assembled in a stepwise fashion using in situ neutralization protocols and HBTU activation.[57] HF or HBr/TFA was used for the activation/deprotection and cyclization/cleavage was performed using a 2% DIEA solution in DMF. The solvent

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Table 1. Crude and Isolated Yields of Cyclic Peptides Using the Safety-Catch Linker 5

entry	product[a]	crude yield[b,e] (%)	HPLC purity of crude (%)	isolated yield[c] (%)
8a	cyclo-[D-Trp-Arg-Thr-(β-Ala)-Phe][16]	62	85	48
8b	cyclo-[L-Trp-Arg-Thr-(β-Ala)-Phe]	64	72	34 (2)[d]
8c	cyclo-[D-Trp-Lys-Thr-(β-Ala)-Phe][16]	70	82	49
8d	cyclo-[Arg-Gly-Asp-(β-Ala)-Phe]	55	40	12 (6)[d]
8e	cyclo-[Arg-Gly-Asp-Gly-(3-Amb)]	50	32	12 (9)[d]
8f	cyclo-[Ser-(β-Ala)-Phe-Ile-Asp]	67[f]	39	25 (7)[d]

[a] All naturally occurring amino acids are the L-isomer unless otherwise stated; β-Ala = β-Alanine; 3-Amb = 3-aminomethylbenzoic acid.[58] [b] Based upon the substitution value of aminomethylpolystyrene resin. [c] After HPLC purification and based upon the substitution value of aminomethylpolystyrene resin. [d] Between brackets is the isolated yield of the cyclic dimer after HPLC purification. [e] Activation was performed using HF/*p*-cresol, 9:1 unless otherwise stated. [f] Activation was performed using HBr/TFA, *p*-cresol.

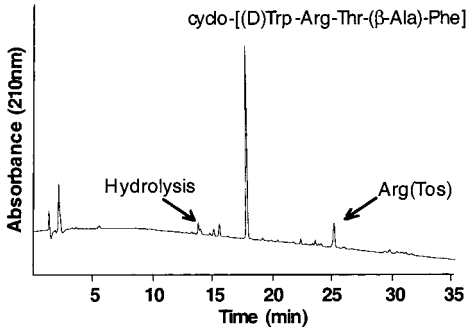

Figure 1. HPLC analysis of crude cyclo-[D-Trp-Arg-Thr-(β-Ala)-Phe]: The solution was loaded directly onto a reversed-phase C-18 (5 μm, 300 Å, 0.46 × 25 cm) HPLC column and analyzed using a linear 10−80% buffer B gradient over 35 min at a flow rate of 1 mL/min: buffer A, H_2O, 0.1% TFA; buffer B, 90% CH_3CN, 10% H_2O, 0.1% TFA.

was subsequently removed under high vacuum and the crude residue analyzed by HPLC and MS. To obtain a more accurate yield of the overall reaction, the target products **8a**−**f** (Table 1) were further isolated from the crude products by HPLC. For **8c**, a treatment with 20% piperidine/DMF of the crude product was first required for the removal of the FMOC protecting group.

Cyclic peptides **8a**−**f** were obtained in varying yields and purity (Table 1). The HPLC chromatogram of the crude cleavage product **7a** (Figure 1) illustrates the quality of the product obtained in this way. For the synthesis of **8b**,**d**−**f**, we obtained a somewhat lower purified yield of the monocyclic peptide and isolated a further 2−9% of cyclo-dimer. The amount of linear peptide in the final crude mixture is minimal as cleavage only takes place through cyclization. A small amount of hydrolysis was observed in earlier experiments but could be attributed to the presence of water in the cleavage solution and/or to slow cyclization of the particular sequence. The hydrolysis was minimized by using freshly dried solvents under an inert atmosphere. Further, we observed 1−3% incomplete detosylation of the arginine residue for peptides **8a**,**b**,**d**,**e**. It is clear that incomplete deprotection can significantly decrease the purity of our crude product. We expect acid-induced, side-chain deprotection to be slower for resin-bound peptides then for cleaved peptides. Optimization of this particular step by selecting suitable protecting groups and deprotection conditions was pivotal to the success of this approach. HBr/TFA is suitable for activation of the linker as is evidenced by the yield and purity of peptide **8f**.

Library Development and Synthesis. The safety-catch linker described here is conceptually ideal for the parallel synthesis of cyclic peptide libraries since it utilizes on-resin side-chain deprotection that, after neutralization and cyclization, gives the desired cyclic peptides in solution. Although the above-described manually synthesized examples prove this concept, an alternative deprotection strategy had to be found since a HF apparatus available for handling large numbers of compounds encountered in parallel synthesis is not usually present in most laboratories. The first choice was HBr/TFA,[59] and early experiments suggested that it gave results similar to those with HF, although with small amounts of partially protected products (Table 1, compound **8f**). However, when HBr/TFA was tried on small libraries, it was found to be unreliable and often required repeated treatments to remove all the protecting groups. Since HBr/TFA is a successful and commonly used resin cleavage/deprotection strategy,[59] it appears that the deprotection of resin-bound peptides is a more difficult process. This may be attributable to the poor resin-swelling characteristics of the deprotection mixture. Even the use of freshly prepared HBr/TFA solutions, or the use of accelerants such as pentamethylbenzene, failed to give reproducible deprotection.

An alternative deprotection strategy that is commonly used in BOC-based peptide synthesis is TFMSA/TFA,[60] and comparisons with HF found that it provided similar on-resin deprotection (Figure 2i,ii). However, as is the case with HBr/TFA, some compromise is required with the choice of protecting groups. For example, cyclohexyl and nitro protecting groups used with the carboxylic and guanidino side chains, respectively, are not effectively cleaved with this procedure.[61] In addition, some of the alternative protecting groups such as Mts or tosyl on arginine are also found to be quite resistant and require longer reaction times at room temperature.[61,62] We found that 10% TFMSA with up to 5 h reaction time at 0 °C, or 1 h at room temperature, was required for complete deprotection. The use of thiol scavengers such as thiocresol or thioanisole was found to be equally effective as *p*-cresol, although not when used in combination with DMS such as in the low TFMSA approach[60] (TFMSA/TFA/DMS/thiocresol (1:5:3:1)). Other alternative side-chain deprotection strategies such as TMSOTf/TFA and TMSBr/TFA[63] were also tried, but were found to be not as effective as TFMSA/TFA. However, when the TFMSA/TFA conditions were applied in a library strategy (see below), a significant side product corresponding, in mass, to the addition of trifluoroacetyl was observed for some

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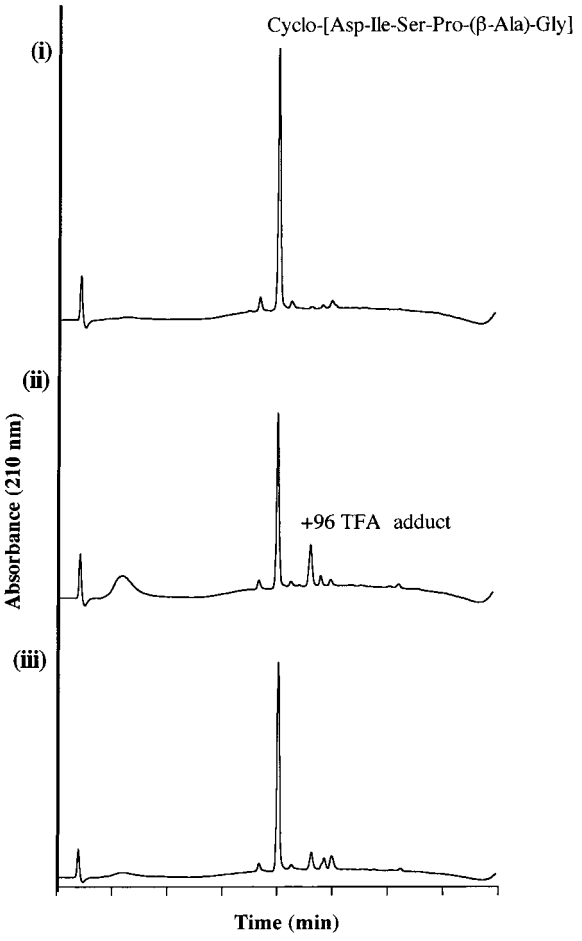

Figure 2. Analytical HPLC of crude cyclic peptide cyclo-[Val-Glu-Asp-(*β*-Ala)-Pro-Tyr]. Deprotection was performed using (i) HF/*p*-cresol, 9:1, 1 h, 0 °C; (ii) TFMSA/TFA/*p*-cresol, 1:10:1, 1 h, rt; (iii) TFMSA/DCM/*p*-cresol, 1:10:1, 1 h, rt.

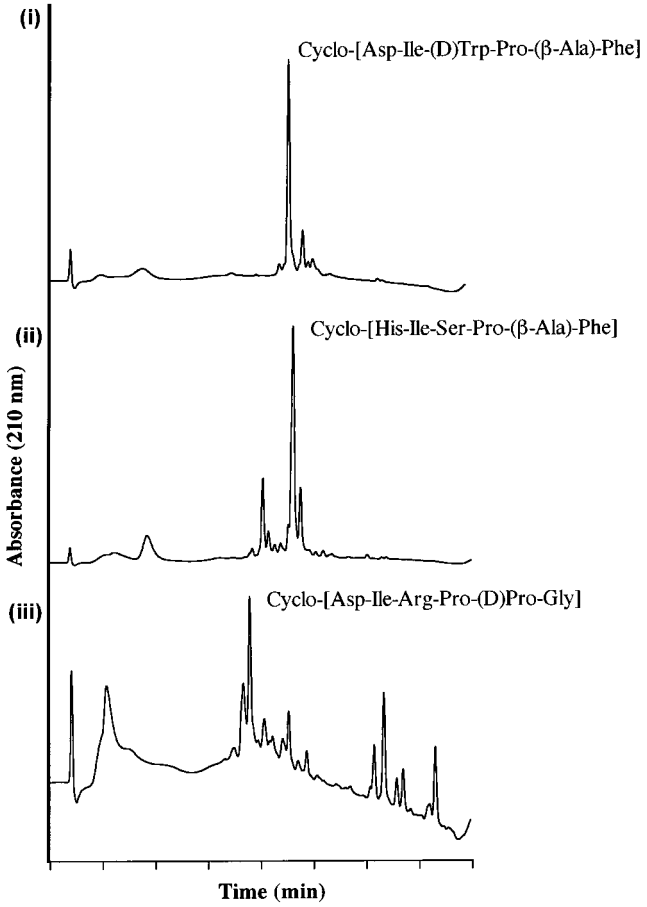

Figure 3. Analytical HPLC of crude cyclic peptide (i) cyclo-[Asp-Ile-D-Trp-Pro-(*β*-Ala)-Phe], (ii) cyclo-[His-Ile-Ser-Pro-(*β*-Ala)-Phe], and (iii) cyclo-[Asp-Il-Arg-Pro-D-Pro-Gly]. (Peak assignments made on the basis of molecular masses as determined by LC-MS.)

sequences. We found that we could circumvent this problem by using 10% TFMSA in DCM for the deprotection/activation step. Comparison of this deprotection/activation strategy with HF and TFMSA/TFA are shown in Figure 2.

A library comprising 432 cyclic peptides were synthesized using an automated robot designed for parallel synthesis. The sequences were either cyclic hexa- or pentapeptdes containing various natural and unnatural amino acids including; Gly, Ala, Ile, Phe, Tyr, Arg, Ser, Thr, Trp, D-Trp, His, Pro, D-Pro, *β*-Ala, 3-methylaminobenzoic acid (3-Amb), isonipecotic acid (Inip), and 6-aminohexanoic acid (Aha). At least four of the amino acids in the sequence corresponded to the natural amino acids. After synthesis and workup, all samples were analyzed by RP-HPLC and MS, and approximately 10% of these samples were also analyzed by LCMS. In addition to the compounds characterized here, a further 24 compounds are given in the Supporting Information. Each sample was then categorized into one of three possibilities based on HPLC and LCMS data (Figure 3). From the data, it was concluded that approximately 15% of the sequences were greater than 75% pure (a typical example is shown from the library in Figure 3i), while 50% were found to be greater than 50% pure (an example is shown in Figure 3ii). While the cyclic peptide in Figure 3iii could be classed as a difficult cyclization sequence. Examples of difficult cyclization sequences are well-known in the literature,[13,64] and we have developed strategies to alleviate these problems.[13,65,66] However, this linker strategy, to date, does not overcome this issue.

Library Optimization. As previously mentioned, for some sequences an impurity corresponding in mass to the addition of trifluoroacetyl onto the cyclic peptide (MH+96 in MS) was found to be a significant side-product in the case of TFMSA/TFA deprotection (Figures 2ii and 4i). This side reaction was observed to occur mostly in serine sequences and can be attributed to acylation of the serine side chain followed by an O → N acyl shift to the terminal amine. This side reaction was first described by Hübener et al.,[67] who observed it in N-terminal threonine sequences under conditions of 99% aqueous TFA. They proposed a mechanism that proceeded through an *O*-trifluoroacetylated intermediate followed by intrachain O → N acyl shift or interchain aminolysis. In our case, the *N*-trifluoroacetyl side product effectively caps the N-terminus so that cyclization can

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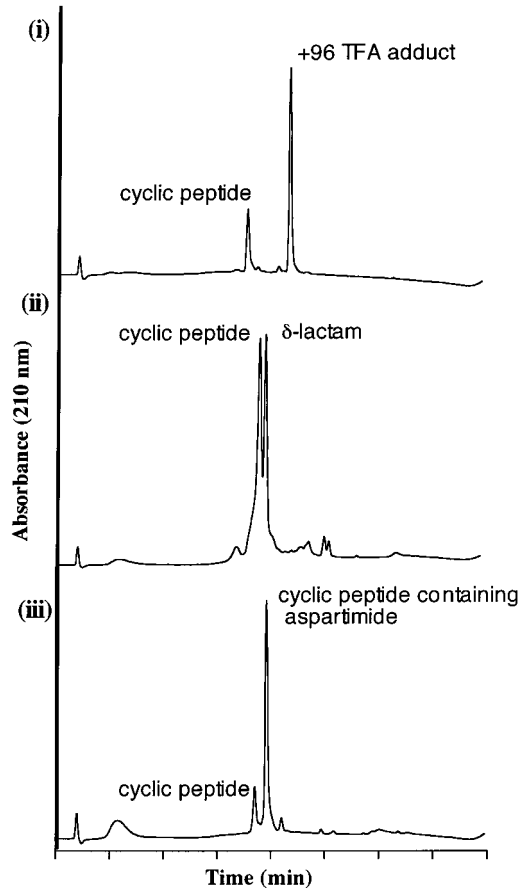

Figure 4. Analytical HPLC of crude cyclic peptides (i) cyclo-[Asp-Ile-Ser-Pro-(*β*-Ala)-Gly], (ii) cyclo-[Asp-Tyr-(*β*-Ala)-Pro-Ala-Arg], and (iii) cyclo-[Tyr-Asp-Gly-(3-Amb)-Gly]. (Peak assignments made on the basis of molecular masses as determined by LC-MS.)

only occur through the serine side chain, and this was shown to be the case since these cyclic products were easily hydrolyzed. The initial serine *O*-trifluoroacetylation must occur during TFMSA/TFA side-chain deprotection since the serine is *O*-benzyl protected during synthesis and this must be followed by the O → N acyl shift upon neutralization with DIEA. Hence, this side product was greatly reduced by replacing the TFA with DCM as the solvent in the deprotection mixture and further by the neutralization of the resin after final BOC deprotection to remove any TFA salts. The use of TFMSA/DCM as the deprotection mixture was also found to work equally well as HF and slightly better than TFMSA/TFA (Figure 2).

Another side reaction that was observed in the synthesis of cyclic peptide libraries was *δ*-lactam formation in sequences with arginine at the C-terminus (Figure 4ii). This side reaction is commonly observed upon activation of unprotected Arg in normal peptide synthesis,[68] and since the activated safety-catch linker is effectively an activated ester, it is not surprising that this side product was observed to be the main product in C-terminal Arg sequences. This side reaction could be easily avoided by simply moving the Arg to a more distant position from the linker. Presumably, this is a consequence of reducing the effective concentration of the guanido group, by distancing the Arg side chain from the active ester, and may also be due to the size of the ring being formed. Positioning the Arg away from the C-terminus allows the more nucleophilic N-terminus to compete. This is more complicated for lysine-containing sequences, these have currently been synthesized with an FMOC-protecting group that is subsequently removed after cyclization (Table 1).

Aspartimide formation was also a significant side-reaction in sequences containing Asp-Gly, Asp-Ser, and Asp-Ala (Figure 4iii). This side product can be easily hydrolyzed to give a mixture of α- and *β*-peptides or if this is deemed undesirable then moving the aspartic acid to the C-terminal position can easily prevent it, as aspartimide formation is not possible with Asp or Glu at the C-terminal position. Importantly, side-chain cyclization of Asp or Glu to the N-terminus is not possible as the only activated ester is the C-terminal carboxy group that is attached to the linker.

Other minor side products that were observed were also cyclic peptides that resulted from various amino acid deletions. These were quite random in nature and are indicative of sequence-related coupling difficulties and are difficult to overcome since monitoring of coupling efficiency is not feasible on a library scale. Likewise the small amount of linear peptide that was detected for several sequences is indicative of sequence-related cyclization difficulties, since the minor amounts of hydrolysis that will occur will appear proportionally larger when cyclization is sluggish or hindered. Detailed LCMS analysis of the products has also revealed the presence of different conformers and oligomers, the presence of which can significantly reduce the apparent crude purity as determined by HPLC.

Conclusions

We have developed and applied a novel "safety-catch" linker for the synthesis of arrays of cyclic peptides. The activation of the safety-catch linker involves debenzylation which is a common orthogonal deprotection strategy employed in peptide chemistry. As a result, it enables simple and mild access to the activated form of the linker allowing head-to-tail cyclization. This has considerable advantages over safety linkers that require alkylation or oxidation that may be problematic to particular amino acid side chains. In addition, the cleavage/cyclization strategy yields cyclic products in a DMF/DIEA solution. Workup is simple solvent removal, allowing the synthesis of very large numbers of compounds using the safety-catch linker **3**. We have currently synthesized >400 discrete cyclic peptides using linker **3**. Finally, as with other handles, our linker is compatible with a wide range of functional supports that are TFA stable.

Experimental Section

General Procedures. Melting points (mp) were determined on a Gallenkamp melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on either a 300 or 500 MHz spectrometer and chemical shifts are reported in *δ* parts per million downfield from tetramethylsilane. Coupling constants (*J*) refer to proton−proton coupling ($J_{H,H}$). ^{13}C NMR spectra were also recorded on the spectrometer at 75.5 MHz. Mass spectra were acquired on a triple-quadrupole mass spectrometer equipped with an ionspray atmospheric pressure ionization source. Samples (10 *μ*L) were injected into a moving solvent (30 *μ*L/min; 50:50 CH_3CN/0.05%TFA) coupled directly

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to the ionization source via a fused silica capillary interface (50 μm i.d. × 50 cm length). Sample droplets were ionized at a positive potential of 5 kV and entered the analyzer through an interface plate and subsequently through an orifice (100−200 μm diameter) at a potential of 80 V. Full scan mass spectra were acquired over a range of 200−2000 Da with a scan step size of 0.1 Da. Accurate mass measurements were acquired on a LCT oa-TOF mass spectrometer equipped with a Z-spray source (Manchester, U.K.). The source was generated in the positive ion electrospray mode, with a declustering potential of 35V. The instrument was operated at a resolution of 5000 fwhm and set to acquire over the mass range *m*/*z* 100−1000 at a speed of 5× spectra per second. Sample analysis, accurate mass measurements and spectral interpretation were performed automatically using a diversity software control. Automated lock mass correction was performed using the protonated molecular ion of [Leu^5] enkephalin at *m*/*z* 566.2771, $C_{28}H_{38}N_5O_7$. Accurate mass measurements of the final products showed good correlation with the expected empirical chemical formuli with mass accuracies of better then 5 ppm. TLC (thin-layer chromatography) was performed on silica gel 60 F254 plates (Merck Art 5735). The chromatograms were viewed under UV light and/or developed with iodine vapor. Preparative column chromatography was effected under pressure, using for normal-phase Merck Kieselgel 60 (Merck Art 7734). Analytical reversed-phase HPLC was run on a C18 column (0.46 × 25 cm), and preparative reversed-phase HPLC was run on a C-18 column (2.2 × 25 cm). Both columns were attached to a HPLC setup fitted with a Holochrome UV detector. Measurements were carried out at $\lambda = 214$ nm.

Materials. BOC-L-amino acids, synthesis grade dimethylformamide (DMF), trifluoroacetic acid (TFA), and diisopropylethylamine (DIEA) were purchased from Auspep (Parkville, Australia). HBTU and BOP were purchased from Richelieu Biotechnologies (Montreal, Canada). AR grade EtOAc, MeOH, CH_2Cl_2, $CHCl_3$, hexane, acetone, and HPLC-grade CH_3CN were all obtained from Laboratory Supply (Australia), HF was purchased from CIG (Australia). Aminomethylpolystyrene resins with a substitution value of 0.26 mmol/g were purchased from Peptide Institute (Osaka, Japan). All other reagents were AR grade or better and were obtained from Aldrich, Fluka, or Bachem AG Switzerland.

Methyl 1-(3,4-Dihydroxyphenyl)propionate.[47] A solution of 3,4-dihydroxyhydrocinnamic acid **4** (10.0 g, 54.9 mmol) and concentrated H_2SO_4 (3 mL) in methanol (250 mL) was heated under reflux overnight. The solvent was evaporated, and the residue was shaken with water and extracted into $CHCl_3$. The combined extracts were dried (Na_2SO_4) and evaporated to give the methyl ester as a pale yellow oil that crystallized on standing (11.2 g, 100%): mp 71.9−74.1 °C (lit.[47] mp 74−76 °C); IR ν_{max} (KBr disk) 3485, 3311, 1712 cm^{-1}; ^{1}H NMR (300 Hz, $CDCl_3$) δ 2.61 (t, $J = 7.5$ Hz, 2H), 2.83 (t, $J = 7.5$ Hz, 2H), 3.69 (s, 3H), 5.40 (br s, 2H), 6.61 (dd, $J = 2.1$, 8.1 Hz, 1H), 6.71 (d, $J = 2.1$ Hz, 1H), 6.77 (d, $J = 8.1$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 30.2, 35.9, 51.9, 115.4, 120.5, 120.5, 133.2, 142.1, 143.6, 174.3; MS M_r 196.0739 (calcd 196.0736).

Methyl 1-(3-Benzyloxy-4-hydroxyphenyl)propanoate and Methyl 1-(4-Benzyloxy-3-hydroxyphenyl)propionate. Benzyl bromide (6.10 mL, 8.72 g, 52.0 mmol) was added to a stirred suspension of methyl 4-(1,2-dihydroxyphenyl)propionate (10.0 g, 51.0 mmol), K_2CO_3 (8.45 g, 61.2 mmol), and a catalytic amount of tetrabutylammonium iodide in DMF (250 mL). The suspension was stirred overnight under an atmosphere of nitrogen. Water (1 L) and 5% HCl (150 mL) were added, and the mixture was extracted with diethyl ether. The combined extracts were washed with water and brine, dried (Na_2SO_4), and evaporated to a brown oil that was purified by flash chromatography (5−20% EtOAc in petroleum) to give a 1:1 mixture of the monobenzyl ethers as a colorless oil (11.5 g, 79%): IR ν_{max} (NaCl thin film) 3446, 1732, 1592, 1514 cm^{-1}; ^{1}H NMR (300 Hz, $CDCl_3$) δ 2.60 (m, 4H), 2.87 (t, $J = 7.8$ Hz, 2H), 2.89 (t, $J = 7.7$ Hz, 2H), 3.67 (s, 3H) 3.68 (s, 3H) 5.08 (s, 2H), 5.09 (s, 2H), 6.67 (dd, $J = 8.2$, 2.1 Hz, 1 H), 6.73 (dd, $J = 8.0$, 1.6 Hz, 1H), 6.81 (m, 2H), 6.82 (d, $J = 8.0$ Hz, 1H), 6.88 (d, $J = 8.2$ Hz, 1H), 7.30−7.50 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 30.3, 30.6, 35.7, 36.0, 51.5, 51.5, 71.0, 71.1, 112.2, 112.4, 114.6, 114.7, 119.6, 121.2, 127.2, 127.3, 127.7, 127.8, 128.2, 128.3, 128.4, 128.6, 132.4, 134.2, 144.2, 144.2, 145.6, 145.8, 173.3, 173.3; ES-MS M_r 286.1 (calcd 286.0).

1-(3-Benzyloxy-4-hydroxyphenyl)propanoic Acid and 1-(4-Benzyloxy-3-hydroxyphenyl)propanoic Acid 5. A solution of lithium hydroxide monohydrate (5.25 g, 125 mmol) in water (150 mL) was added to a stirred solution of the mixture of methyl esters (11.95 g, 41.7 mmol) in THF (150 mL). The solution was stirred overnight, and then the THF was evaporated off and the residue was diluted with water (150 mL) and acidified to pH 3 with 5% HCl. The mixture was extracted with $CHCl_3$ (3 × 350 mL), and the combined extracts were dried (Na_2SO_4) and evaporated to a brown oil that solidified on standing. This was taken up in EtOAc and passed through a short silica column. Evaporation of the eluent gave the product as a tan solid **5** (11.12 g, 98%): IR ν_{max} (KBr disk) 3533, 3471, 3300−2600, 1718, 1699, 1515 cm^{-1}; ^{1}H NMR (300 Hz, $CDCl_3$) δ 2.66 (m, 4H), 2.90 (t, $J = 7.6$ Hz, 2H), 2.91 (t, $J = 7.7$ Hz, 2H), 5.09 (s, 2H), 5.10 (s, 2H), 6.69 (dd, $J = 8.3$ Hz, 2.1 Hz, 1H), 6.75 (dd, $J = 8.1$, 2.0 Hz), 6.83 (d, $J = 1.9$ Hz, 1H), 6.84 (d, $J = 2.0$ Hz, 1H), 6.87 (d, $J = 8.4$ Hz, 1H), 6.90 (d, $J = 8.2$ Hz, 1H), 7.30−7.50 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 29.9, 30.2, 35.7, 35.9, 71.1,71.2, 112.3, 112.4, 114.6, 114.7, 119.6, 121.2, 127.2, 127.3, 127.7, 127.8, 128.3, 128.4, 128.7, 132.0, 136.2, 144.3, 145.6, 145.8, 179.2, 179.2; ES-MS M_r 272.1 (calcd 271.9).

1-[3-Benzyloxy-4-(BOC-Phe-O)phenyl]propanoic Acid and 1-[4-Benzyloxy-3-(BOC-Phe-O)phenyl]propanoic Acid. Triethylamine (1.40 mL, 1.01 g, 10.0 mmol) and ethyl chloroformate (0.96 mL, 1.085 g, 10.0 mmol) were added to a stirred, chilled (−8 °C) solution of BOC-Phe-OH (2.65 g, 10.0 mmol) in dichloromethane (20 mL). The solution was stirred for 20 min at −8 °C, during which time a precipitate formed. A solution of a regioisomeric mixture of benzyloxy acids **5** (2.86 g, 10.0 mmol) and triethylamine (1.40 mL, 1.01 g, 10.0 mmol) in dichloromethane (20 mL + 5 mL rinse) was then added dropwise. The resulting solution was stirred at −5 to 0 °C for 2 h, washed with 10% citric acid and brine, dried (Na_2SO_4), and evaporated to a syrup. This was dissolved in a small amount of 1:1 ethyl acetate/petroleum ether and passed through a short silica column. Evaporation of the eluent gave the mixture of title carboxylic acids as a colorless syrup (4.47 g, 86%): ^{1}H NMR (300 Hz, $CDCl_3$) δ 2.66 (m, 4H), 2.90 (t, $J = 7.6$ Hz, 2H), 2.91 (t, $J = 7.7$ Hz, 2H), 5.09 (s, 2H), 5.10(s, 2H), 6.69 (dd, $J = 8.3$ Hz, 2.1 Hz, 1H), 6.75 (dd, $J = 8.1$, 2.0 Hz), 6.83 (d, $J = 1.9$ Hz, 1H), 6.84 (d, $J = 2.0$ Hz, 1H), 6.87 (d, $J = 8.4$ Hz, 1H), 6.90 (d, $J = 8.2$ Hz, 1H), 7.30−7.50 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 29.9, 30.2, 35.7, 35.9, 71.1,71.2, 112.3, 112.4, 114.6, 114.7, 119.6, 121.2, 127.2, 127.3, 127.7, 127.8, 128.3, 128.4, 128.7, 132.0, 136.2, 144.3, 145.6, 145.8, 179.2, 179.2; ES-MS M_r 519.1 (calcd 519.2).

1-[3-Benzyloxy-4-(BOC-Gly-O)phenyl]propanoic Acid and 1-(4-Benzyloxy-3-(BOC-Gly-O)phenyl]propanoic Acid. Triethylamine (1.40 mL, 1.01 g, 10.0 mmol) and ethyl chloroformate (0.96 mL, 1.085 g. 10.0 mmol) were added to a stirred, chilled (−20 °C) solution of BOC-Gly-OH (1.75 g, 10.0 mmol) in dichloromethane (20 mL). The solution was stirred for 20 min at −10 to −15 °C, during which time a precipitate formed. A solution of regioisomeric mixture of benzyloxy acids **5** (2.86 g, 10.0 mmol) and triethylamine (1.40 mL, 1.01 g, 10.0 mmol) in dichloromethane (20 mL + 5 mL rinse) was then added dropwise. The resulting solution was stirred at −5 to 0 °C for 2 h, washed with 10% citric acid (2 × 10 mL) and brine (10 mL), dried (Na_2SO_4), and evaporated to a syrup. This was dissolved in a small amount 1:1 ethyl acetate/petroleum ether and passed through a short silica column. Evaporation of the eluent gave the mixture of title carboxylic acids as a colorless syrup (3.99 g, 93%): ^{1}H NMR (300 Hz, $CDCl_3$) 1.47 (s, 9H) 2.65 (t, $J = 6.6$, 2H), 2.85−2.95 (m, 2H), 4.15−4.17 (m, 2H), 5.07 (s, 2H), 5.08−5.15 (m, 1H), 6.66−7.46 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) 28.3, 29.6, 30.4, 35.3 35.6, 42.3, 70.7, 71.3, 80.1, 155.6, 178.0, 178.4; ES-MS M_r 430.4 (calcd 430.2).

1-[3-Benzyloxy-4-(*N*-*tert*-butoxycarbonyl)aspartyloxy]-phenylpropanoic Acid and 1-(4-Benzyloxy-3-(*N*-*tert*-bu-

toxycarbonyl)aspartyloxy]phenylpropanoic Acid 3c. The symmetric anhydride of BOC-Asp(OBzl) was prepared by adding DIC (783 μL, 5 mmol) to a stirred solution of BOC-Asp(OBzl) (3.23 g, 10 mmol) in DCM (5 mL). The solution was then added to H-Gly-Leu-Leu-aminomethylpolystyrene resin (5.00 g, 0.233 mmol/g) previously derivatized with benzyloxy acids **5** (5.7 g, 1.3 mmol) using in situ neutralization/HBTU activation protocols for BOC chemistry.[57] Solid DMAP (305 mg, 2.5 mmol) was then added to the solution and shaking continued for 2 h. The resin was then filtered and washed with DMF.

General Procedure for Solid-Phase Peptide Synthesis. The linear peptides **6a**−**f** were synthesized in stepwise fashion by established methods using in situ neutralization/HBTU activation protocols for BOC chemistry.[57] The mesitylene-2-sulfonyl protecting group was used for the Arg residue, fluorenylmethyl carbonate for lysine, the benzyl ether for the Thr residue and Ser residue, and the benzyl ester for the Asp residue. 3-Aminomethylbenzoic acid (3-Amb) was synthesized as described by a previously reported method.[58] Coupling reactions were monitored by quantitative ninhydrin assay and were typically >99.9%. After chain assembly was complete, the removal of the protecting groups was conducted using either HF/*p*-cresol, 11 mL, 10:1, for 1 h at −5 °C or HBr/TFA (5 mL), *p*-cresol (0.5 mL), for 1 h at rt. After activation/deprotection, the resin was washed with anhydrous ether, filtered, and dried.

Cyclo-[D-Trp-Arg-Thr-(β-Ala)-Phe] 8a.[15] Cyclization was performed by treatment of the resin **7a** (200 mg, 0.202 mmol/g) using a 2% solution DIEA/DMF (4 mL). The solvent was removed under reduced pressure to give a white solid (16.6 mg, 62%). The peptide **8a** was purified by semipreparative HPLC (10−70% B over 60 min) and lyophized to give a white powder (12.8 mg, 48%): ES-MS M_r 662.3417 (calcd 662.3415). This cyclic peptide was synthesized using a different methodology.[15] Both peptides had the same retention time by HPLC.

Cyclo-[L-Trp-Arg-Thr-(β-Ala)-Phe] 8b. Cyclization was performed by treatment of the resin **7b** (205 mg, 0.202 mmol/g) using a 2% solution DIEA/DMF (4 mL). The solvent was removed under reduced pressure to give a white solid (17.5 mg, 64%). The peptide **8b** was purified by semipreparative HPLC (10−70% B over 60 min) and lyophized to give a white powder (9.3 mg, 34%). NMR was consistent with structure: ES-MS M_r 662.3405 (calcd 662.3415). Also isolated was the cyclic dimer (0.4 mg, 2%): ES-MS M_r 1224.7 (calcd 1224.7).

Cyclo-[D-Trp-Lys-Thr-(β-Ala)-Phe] 8c.[15] Cyclization was performed by treatment of the resin **7c** (220 mg, 0.194 mmol/g) using a 2% solution DIEA/DMF (5 mL). The solvent was removed under reduced pressure to give a white solid (26.1 mg, 70%). This powder was treated with a 20% solution of piperidine in DMF at r.t. for 2h. Solvent was removed under reduced pressure to give a white solid. The peptide **7c** was purified by semipreparative HPLC (10−70% B over 60 min) and lyophised to give a white powder (13.0 mg, 49%): ES-MS M_r 634.3353 (calcd 634.3351). This cyclic peptide was synthesized using a different methodology.[15] Both peptides had the same retention time by HPLC.

Cyclo-[Arg-Gly-Asp-(β-Ala)-Phe] 8d. Cyclization was performed by treatment of the resin **7d** (180 mg, 0.207 mmol/g) using a 2% solution DIEA/DMF (3.5 mL). The solvent was removed under reduced pressure to give a white solid (11.6 mg, 55%). The peptide **8d** was purified by semipreparative HPLC (0−60% B over 60 min) and lyophised to give a white powder (2.6 mg, 12%): ES-MS M_r 547.2629 (calcd 547.2648). Also isolated was the cyclic dimer (1.3 mg, 6%): ES-MS M_r 1093.7 (calcd 1093.5).

Cyclo-[Arg-Gly-Asp-Gly-(3-Amb)] 8e.[56] Cyclization was performed by treatment of the resin **7e** (524 mg, 0.208 mmol/g) using a 2% solution DIEA/DMF (10 mL). The solvent was removed under reduced pressure to give a white solid (103 mg, 50%).. The peptide **8e** was purified by semipreparative HPLC (10−70% B over 60 min) and lyophised to give a white powder (24.5 mg, 12%): ES-MS M_r 519.2 (calcd 519.2). NMR is consistent with structure and was identical to that previously reported in the literature.[56] Also isolated was the cyclic dimer (19 mg, 9%): ES-MS M_r 1037.4 (calcd 1037.4).

Cyclo-[Ser-(β-Ala)-Phe-Ile-Asp] 8f. Cyclization was performed by treatment of the resin **7f** (180 mg, 0.207 mmol/g) using a 2% solution DIEA/DMF (4 mL). The solvent was removed under reduced pressure to give a white solid (13.8 mg, 67%). The peptide **8f** was purified by semipreparative HPLC (10−70% B over 60 min) and lyophised to give a white powder (5.1 mg, 25%): ES-MS M_r 534.2564 (calcd 534.2638). Also isolated was the cyclic dimer (1.4 mg, 7%): ES-MS M_r 1067.7 (calcd 1067.5).

Attachment of the Safety-Catch Linker to Resin. 3-(1-Hydroxy-2-benzyloxyphenyl)propanoic acid (813 mg, 3 mmol) and HBTU (1.137 g, 3 mmol) were dissolved in DMF (6 mL). To this was added DIEA (690 μL, 4 mmol) and the resulting solution added to precoupled H-Gly-Leu-Leu-aminomethylpolystyrene (6.0 g, 0.233 mmol/g). The resin was shaken for 1 h or until >99% completion as determined by quantitative ninhydrin test. The eluant was filtered off and the resin washed with DMF (4 × 12 mL).

General Procedure for the Acylation of the Safety-Catch Linker. The symmetric anhydrides of BOC-amino acids were prepared by adding DIC (939 μL, 6 mmol) to a stirred solution of BOC-amino acids (12 mmol) in DCM (6 mL). The solution was then added to H-Gly-Leu-Leu-aminomethylpolystyrene resin previously derivatized with benzyloxyacids **2**. Solid DMAP (∼50 mg) was then added to the solution and shaking continued for 3 h. The resin was then filtered and washed with DMF (4 × 12 mL) and then DCM (4 × 12 mL).

General Procedure for the Automated Synthesis of Linear Peptides. Approximately 100 mg of resin was distributed to each of the reaction block wells by pipetting a slurry of the resin in DMF/DCM (3:1). The peptides was then assembled by a combinatorial chemistry apparatus suited for parallel synthesis using in situ neutralization/HBTU activation protocols for BOC chemistry.[57] The resin was initially washed with DCM (2 × 1 mL) and the BOC protecting group removed using a 40% solution of TFA in DCM (2 × 1 mL). The resin was further washed with DCM (1 mL) and then DMF (4 × 1 mL). To each well was added a solution of the appropriate amino acid (0.3 mmol) and HBTU (142.8 mg, 0.3 mmol) in DMF (1 mL). DIEA (50 μL, 0.3 mmol) was then added to each reaction mixture. The reaction block was shaken for 1 h and emptied and the resin washed with DMF (3 × 1 mL). Deprotection, coupling, and washing cycles were repeated stepwise to assemble the linear peptides.

General Procedure for Deprotection and Cyclization of Linear Peptides. After the final BOC deprotection of the linear peptide, the resin was dried under nitrogen for 30 min. A solution of TFMSA/TFA/*p*-cresol (1:10:1) was prepared by the dropwise addition of TFMSA (6 mL) to a stirred solution of *p*-cresol (6 mL) in TFA (60 mL) at −5 °C. One millilter of this solution was then added to each of the reaction block wells, and the resin was shaken for 1 h. The resin was then washed with TFA (2 × 1 mL), DCM (1 × 1 mL), and DMF (3 × 1 mL). A solution of DIEA (2%) in DMF was then added to each of the reaction wells, and the reaction block was shaken overnight. The eluant from the reaction block was collected into a deep-well titer plate, the resin rinsed with DMF, and the eluant collected into a separate titer plate. The solvent was removed by centrifuge evaporation, and the product was dissolved in 0.1% TFA/45% CH_3CN/55% H_2O. Further drying resulted in crude cyclic peptides.

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Supporting Information Available: Library synthesis and ES-MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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