Synthesis of Difficult Cyclic Peptides by Inclusion of a Novel Photolabile Auxiliary in a Ring Contraction Strategy

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Abstract: Cyclic peptides comprise a large and important class of biologically active molecules. They are generally synthesized through amide bond-forming reactions of the *C*- and *N*- termini under high dilution conditions. Yields of such processes are highly dependent on the size of the ring being formed and on the particular amino acids of the linear precursor, giving rise to the well-known sequence-dependent effect of cyclization. To overcome this problem, we have developed a peptide cyclization strategy that proceeds through a ring closure/ring contraction process. The linear peptide Ala-Phe-Leu-Pro-Ala, which does not generate monocyclic product under conventional cyclization conditions, was used as a model to probe a range of auxiliaries. This has led to the development of a new photolabile peptide cyclization auxiliary. The 6-nitro-2-hydroxybenzyl group is readily and quantitatively introduced at the *N*-terminus via a reductive alkylation. Cyclization of the auxiliary-peptide initially proceeds through a cyclic nitrophenyl ester that preorganizes the peptide for lactamization. As the *C*- and *N*- termini are in close proximity, lactamization is achieved via an intramolecular *O*−*N* acyl transfer step to produce the *N*-substituted target cycle. The auxiliary is then removed by mild photolysis to produce the target cyclic peptide, cyclo-[Ala-Phe-Leu-Pro-Ala], in good yield. This strategy should find further useful applications in the assembly of libraries of small cyclic peptides.

Introduction

In the linear form, bioactive peptides can assume millions of different conformations, very few of which are able to bind to their receptor. To assess the important structural and dynamic properties that are critical for the biological potency and selectivity of peptides, conformational constraints are often introduced, typically through cyclization.[1−6] Such cyclic molecules may exist in more clearly defined conformations and are appealing from a drug lead discovery perspective.[7] If activity is maintained or enhanced in these cyclic peptides, structural information is sought, by NMR, X-ray or molecular modeling, and used to guide the development of therapeutic drugs.[8−10] In addition, cyclization generally promotes an increase of metabolic stability and bioavailability of peptides.[11,12]

As the side chains are generally considered to be the main mediators for receptor interaction,[1] cyclization is preferably accomplished between the C- and N-termini. Whereas the synthesis of linear peptides generally proceeds well, head-to-tail cyclization is often troublesome, especially for small peptides of less than seven residues in length.[13] The primary reason for ineffective cyclization originates from a sequence-related inefficiency to bring the termini together for head-to-tail cyclization. Since peptide bonds contain strong π-character and preferentially adopt a trans conformation, linear peptides prefer more extended conformations. This places the terminal carboxylic acid and amine functional groups in remote positions that are unfavorable for cyclization. Incorporation of turn-inducing elements such as Gly, Pro, or D-amino acids are known to enhance cyclization yields.[14] For linear peptides of 4−6 residues that do not contain amino acids that stabilize turn structures, slow cyclizations lead to side reactions such as cyclodimerization and epimerization.[15]

We are interested in developing chemical strategies that facilitate the assembly of libraries of small cyclic peptides.[16] To address the sequence-related inefficiency of small peptide cyclizations, we have aimed at developing auxiliary strategies

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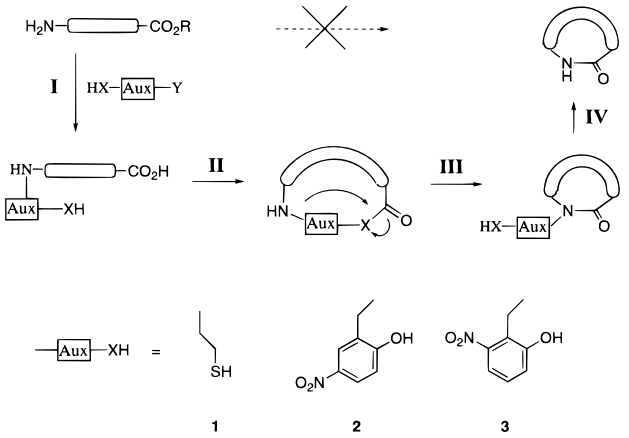
Scheme 1. A Peptide Cyclization Auxiliary Strategy[a]



[a] The strategy involves (I) introduction of the auxiliary at the N-terminus; (II) ring closure; (III) ring contraction; and (IV) removal of the auxiliary. The auxiliaries **1**, **2**, and **3** were employed in this work.

that preorganize the N- and C-termini of a single chain for head-to-tail cyclization. In one strategy (Scheme 1) an auxiliary (Aux) carrying a hydroxy or thiol functionality (XH) is introduced at the N-terminus of the peptide (Step **I**) and extends the sequence by several atoms. Activation of the C-terminus initially results in the formation of a more accessible cyclic ester (Step **II**), thereby bringing the N- and C-termini of the peptide closer in space. An *X*-to-*N* acyl transfer (Step **III**) results in a ring contraction to generate the target ring size, and removal of the auxiliary (Step **IV**) produces the desired monocyclic product.

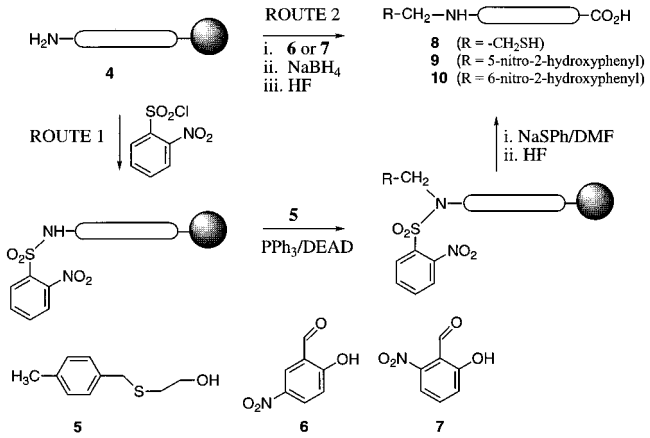
A suitable auxiliary must fulfil the following criteria: (i) introduction of the auxiliary at the N-terminus of the peptide should be facile and independent of the nature of the *N*-terminal residue; (ii) to favor cyclization over oligomerization (step **II**), the auxiliary must be sufficiently flexible to allow the N- and C-termini, that is, the hydroxy/thiol functionality of the auxiliary and the activated carbonyl at the C-terminus to meet (iii) as there is no need to isolate the intermediate cyclic ester, rapid ring contraction is preferred to avoid competitive ring opening side reactions. Therefore the transition state geometry for this acyl-transfer step should not be impeded by ring strain or steric bulk caused by the extra atoms of the auxiliary. A more reactive auxiliary-ester in the intermediate cyclic product should further enhance the rate of the desired rearrangement but may increase the rate of unwanted side reactions such as hydrolysis; (Intramolecular acyl transfers have been extensively studied by Kemp et al.[22,34−37] although not in a cyclization strategy. The chief parameters that influence the rate of transfer are the ring size of the transition state,[34,37] and the correlated steric and electronic effects of the auxiliary.[35]) (iv) removal of the auxiliary should occur efficiently via orthogonal chemistry, not affecting the peptide functionalities.

We now wish to report a novel photolabile auxiliary that fulfils the criteria and has enabled the formation of small cyclic peptides from "difficult" linear precursors in good yield and purity.

Results

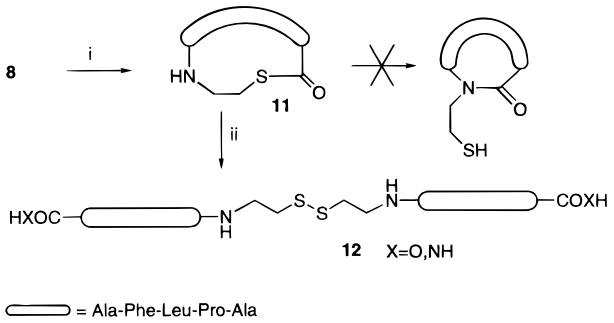
Cyclization of Ala-Phe-Leu-Pro-Ala. We selected the linear peptide Ala-Phe-Leu-Pro-Ala for evaluating the ring contraction strategy. Cyclization of this unsubstituted peptide has been reported to yield cyclic dimers (decapeptide) and higher oligomers but no cyclopentapeptide[15] and thus serves as an excellent model to examine new peptide cyclization auxiliaries.

Scheme 2. Two Reaction Routes for the Introduction of the Auxiliaries **1**-**3**[a]



[a] Route 1 via a Fukuyama approach using alcohol **5**; route 2 via reductive amination using the benzaldehydes **6** and **7**.

Scheme 3. Cyclization of the *N*-Mercaptoethanyl Peptide **8**[a]



[a] (I) 3 equiv of BOP/5 equiv of DIEA, 3 h at room temperature; (II) 0.1 M NH_4HCO_3.

As a control experiment, we attempted to cyclize the unsubstituted linear peptide (Ala-Phe-Leu-Pro-Ala) using standard cyclization conditions (1 mM in DMF [*N*,*N*-dimethylformamide], 3 equiv of BOP [benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate], 5 equiv of DIEA [diisopropylethylamine], 3 h at room temperature). As expected from the previously reported results, only cyclic dimer and some trimer were obtained but no target monocyclic product.

Evaluation of Ethanethiol Auxiliary 1. We initially evaluated an ethanethiol auxiliary **1**. This auxiliary was introduced via an on-resin Fukuyama synthesis[17−19] (Scheme 2, Route 1). An *O*-nitrobenzene sulfonamide (ONBS), prepared from the corresponding sulfonyl chloride and the primary amine **4**, was alkylated using *S*-(4-methylbenzyl)-2-thioethanol **5** under Mitsunobu-like conditions. The ONBS-group was then removed using sodium thiophenoxide (PhSNa) in DMF, prior to HF cleavage and deprotection of the thiol functionality.

Cyclization Using Ethanethiol Auxiliary 1. Whereas cyclization of the parent peptide only generates oligomeric material, cyclization of the *N*-ethanethiol derivative **8** (Scheme 3) under the same conditions yielded only the monocyclic product **11** (45% isolated yield), as determined by mass spectral analysis (correct molecular weight and isotope distribution). No dimeric (or other oligomeric) products were found in the crude reaction mixture.

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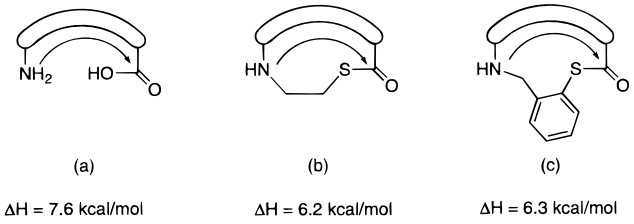
Scheme 4. Estimated Activation Energies for Lactam Formation from the Linear Unsubstituted Peptide (a) and from the Cyclic Auxiliary Esters (b,c)



The monocyclic product has the thioester structure, as confirmed by saponification of the monocyclic product (**11)** in NH_4HCO_3, which generated disulfides **12** of the linear peptide amides and acids. Attempts to force ring contraction by heating (65 °C) the isolated ester in organic solvents (DMF, dioxane) in the presence of base (DIEA, DBU {1,8-diazabicyclo[5.4.0]-undec-7-ene}), or heating in aqueous buffers (pH 4−8) failed. The ester either remained unchanged or hydrolyzed to the linear peptide.

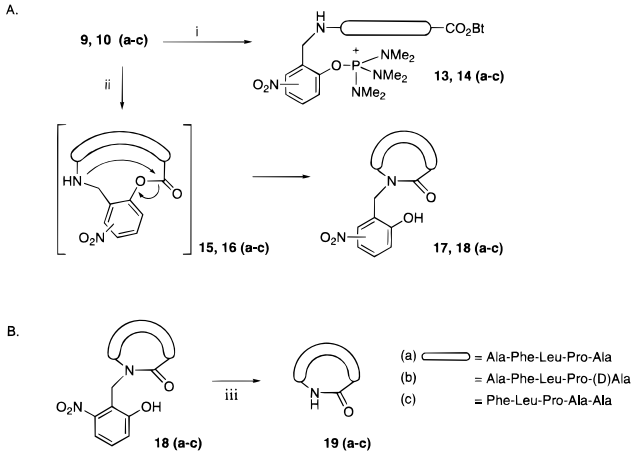
This failure to ring contract may be due to steric hindrance in the transition state caused by the auxiliary. To evaluate this we undertook a series of molecular modeling investigations.

Evaluation of the Transition-State Geometry. The proposed ring contraction approach involves a bicyclic transition state comprised of a larger peptidic ring as determined by the length of the peptide and a smaller ring determined by the size of the auxiliary. To evaluate the ring strain and steric crowding of this transition state for the proposed auxiliaries, we undertook Monte Carlo conformational searching using the Amber* force field in the Macromodel program, using a procedure similar to Cavelier-Frontin.[20] Tetra-alanine was selected as a model peptide. The transition state of the cyclization reaction was simulated by applying a series of distance constraints that have been derived from experimental data.[21] Bürgi *et al.* had previously used structural regularities in crystal structures to map the reaction coordinates for the attack of a nucleophilic nitrogen atom and a carbonyl group. Applying this transition-state geometry to both unsubstituted peptides and auxiliary-containing peptides followed by extensive exploration of their conformational space allowed the calculation of the low-energy conformations that are able to adopt the transition-state geometry. The activation energy required for achieving this transition geometry was then calculated by subtracting the energy of the "lowest" conformation found in an unconstrained conformational search from the energy of the "lowest" transition-state conformation (Scheme 4).

The calculated activation energies for amide bond formation in the cyclization of the unsubstituted linear peptide (Scheme 4a) and the auxiliary-linked equivalents (Scheme 4b,c) are very similar. The results suggest that the additional five- or six-membered ring system (from the ethanethiol or hydroxybenzyl auxiliaries respectively) in the bicyclic transition state does not introduce significant steric strain.

Nitrobenzyl Auxiliaries 2, 3. Following the above findings, we concluded that the ring contraction of the cyclic alkylthioester **11** (Scheme 3) was mostly impeded by a low reactivity of the alkylthioester toward secondary amines rather than by a constrained transition-state geometry. We decided to examine the 2-hydroxynitrobenzyl auxiliaries **2** and **3** in the ring contraction approach, as initial cyclization would generate a more reactive nitrophenylester with significantly improved acyl transfer kinetics.[22,23] Moreover, because of the similarity with photolabile *ortho*-nitrobenzyl linkers, we expected that photolysis would enable removal of auxiliary **3**.[24]

Scheme 5. Cyclization of Auxiliary Containing Peptides **9-10** (A) and Formation of the Target Cyclic Peptides **19** (B)[a]



[a] (I) 3 equiv of BOP/5 equiv of DIEA, 3 h at room temperature; (II) 1 equiv of BOP/2 equiv of DIEA, 3 h rt; 10 equiv of DIEA, 12 h rt or 1 h at 65°C; (III) hν (366 nm).

The auxiliaries **2** and **3** were readily introduced by a two-step reductive alkylation[25] of the *N*-terminal primary amine **4** with aldehydes **6** or **7** (Scheme 2, Route 2). (5-Nitro-2-hydroxy benzaldehyde is commercially available, while 6-nitro-2-hydroxy benzaldehyde is readily synthesized via a Duff reaction.[38]) A small excess of aldehyde was initially used to generate the intermediate imine in quantitative yields (5 min). Reduction of the imine after removal of the excess aldehyde was then accomplished using $NaBH_4$ (5 min). HF cleavage produced the *N*-terminal substituted peptides **9** and **10** in high yield and excellent purity. Racemization or incomplete alkylation of the *N*-terminal amino acid was not observed.

Cyclization Using 5-Nitro-2-hydroxybenzyl Auxiliary 2. Cyclization of peptide **9a** under standard conditions initially yielded two monocyclic products as well as significant amounts of a side product **13a** (Mr, 812 Da), caused by reaction of the phenol functionality with excess BOP in the reaction mixture (Scheme 5, A). By adjusting the amount of activating reagent and base, formation of this side product was completely avoided. The reaction conditions were further optimized by altering the temperature and amount of base after an initial cyclization period and monitoring the formation of monocyclic products by LC/MS (liquid chromatography/mass spectrometry) analysis. The best results were obtained when, after 3 h of reaction (1 mM in DMF, 1 equiv of BOP, 2 equiv of DIEA, rt), excess DIEA (10 equiv) was added and the mixture was left standing for 24 h or heated to 65 °C for 1 h. The HPLC profile of the crude product is depicted in Figure 1B. The main product (50% isolated yield) was characterized by NMR, ES-MS (electrospray mass spec-

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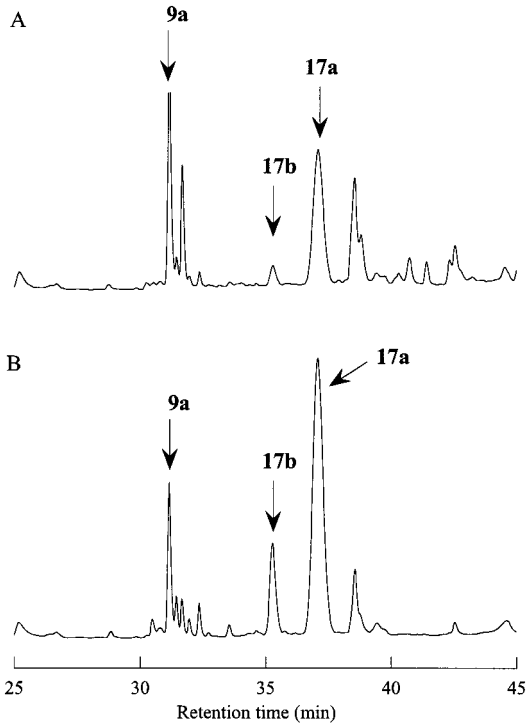

Figure 1. HPLC analysis of cyclization of linear peptide **9a** (A) after 3 h at room temperature and (B) 1 h heating to 65 °C in the presence of excess DIEA. The solutions were dried under high vacuum, were dissolved in 50% aqueous acetonitrile, and were loaded directly onto a Vydac reversed-phase C-18 (5 μm, 300 Å, 0.46 × 25 cm) HPLC column. The products were separated using a linear 0−80% buffer B gradient over 40 min at a flow rate of 1 mL/min.

trometry), and chiral amino acid analysis as the all-L target monocyclic product **17a**. A ^{1}H NMR absorption at 11.5 ppm confirmed that the product contained the free hydroxy substituent and thus did not have the ester structure but rather the target cyclic amide structure. Further, a small amount of the C-terminally racemized product **17b** (see Figure 1B) was also isolated. A chiral amino acid analysis of the product displayed the presence of a D-Ala residue.

In an attempt to isolate the intermediate cyclic ester **15a**, the reaction mixture was analyzed after the initial 3 h cyclization period by HPLC (Figure 1A) and LC/MS. The mixture contained linear peptide **9a** and monocyclic products **17a** and **17b**, but no monocyclic ester was found. The *p*-nitrophenyl ester presumably hydrolyzes in the aqueous workup to the linear peptide.

Cyclization Using 6-Nitro-2-hydroxybenzyl Auxiliary 3. As the 5-nitro-2-hydroxybenzyl auxiliary is not readily removed after cyclization, we examined the 6-nitro-2-hydroxybenzyl auxiliary peptide **10a** toward cyclization. The *ortho*-nitro substituent, while maintaining a similar activation effect on the ring contraction of the cyclic intermediate **16a** (compared to **15a**), has the added benefit that it should render the auxiliary photolabile. The linear peptide **10a** was synthesized and treated as described above for the 5-nitro-2-hydroxy derivative (Scheme 5A). Thus cyclization (at 1 mM in DMF, 1 equiv of BOP/2 equiv of DIEA) was performed at room temperature for 3 h, followed by addition of excess DIEA (10 equiv) and heating to 65 °C for 1 h. The major product was isolated in 39% yield and characterized by NMR and chiral amino acid analysis as the all-L cyclo-pentapeptide **18a**. A small amount of the C-terminal racemized cyclic product (containing a D-Ala) **18b** was also isolated.

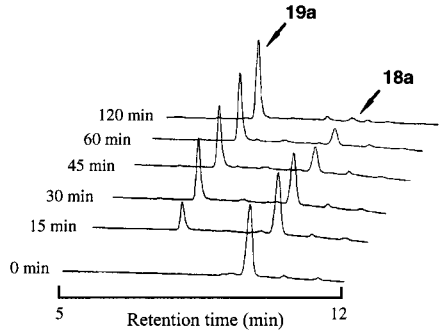

Figure 2. HPLC analysis of the photolysis of cyclic peptide **18a** at timed intervals. A 0.15 mM solution of peptide **18a** in MeOH/1% AcOH was photolyzed using a standard UV lamp, and at different time intervals, small aliquots were injected onto a Zorbax reversed-phase C-18 (3 μm, 300 Å, 0.21 × 5 cm) HPLC column. The products were separated using a linear 0−80% buffer B gradient over 10 min at a flow rate of 200 μL/min (detection at 214 nm).

Similarly *N*-(6-nitro-2-hydroxybenzyl)Phe-Leu-Pro-Ala-Ala **10c** was assembled and cyclized as above. The all-L cyclo pentapeptide **18c** was isolated in 45% yield.

Removal of the Auxiliary. Cyclic peptide **18a** was then subjected to photolysis at 366 nm, using a standard UV lamp, in a range of solvent conditions. In most solvents (MeOH, MeOH/AcOH, THF/AcOH, dioxane) (AcOH = acetic acid; MeOH = methanol; THF = tetrahydrofuran) the nitrobenzyl substituent on the backbone nitrogen is readily removed to generate the target cyclic peptide **19a** (Scheme 5, B). Figure 2 illustrates the clean and efficient conversion (**18a** to **19a**). The cyclic product was characterized by chiral amino acid analysis and ^{1}H NMR. The spectral data are in good agreement with the reported data.[15] Further, an independent sample of cyclic peptide, prepared from the cyclization of Phe-Leu-Pro-Ala-Ala according to Schmidt *et al.*,[15] coeluted with the product obtained from photolysis.

The same product **19a** was obtained from photolysis of the regio analogue **18c**. The racemized cyclic product **18b** was photolyzed and similarly produced the unsubstituted D-Ala containing product **19b**, which coeluted with an independently synthesized sample.

The rate of photolysis is highly dependent on the presence of acid or base. In acidic environment (MeOH, MeOH/AcOH, THF/AcOH), photolysis is fast and clean, whereas in basic environment (THF dried on KOH, or in the presence of 10% hydrazine), photolysis is very slow (Figure 3).

Discussion

The synthesis of cyclic peptides generally involves an entropically disfavored head-to-tail cyclization. The choice of linear precursor for cyclization can dictate the level of success of the synthesis, and consequently, the cyclization position should be carefully chosen according to a number of simple guidelines: (i) the cyclization site should not be sterically encumbered, (ii) if possible cyclization should occur between a D and an L-residue,[13] (iii) turn-inducing elements such as Gly and Pro favor cyclization,[15] and (iv) intrachain hydrogen bonds can facilitate peptide cyclization.[26] However, adhering to these simple guidelines does not guarantee that any cyclic product will be obtained. For example, the control sequence used in this study Ala-Phe-Leu-Pro-Ala abides by these simple rules, yet no cyclic monomeric product is obtained upon cyclization. In addition, in the development of a backbone linker that is

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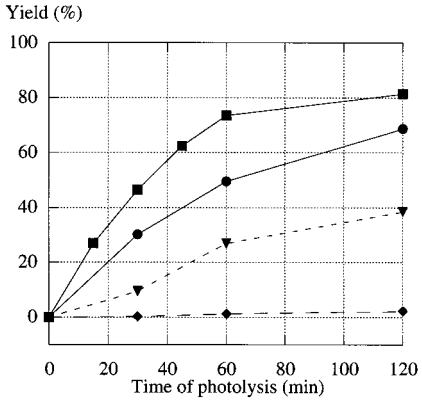

Figure 3. Yield of photolysis of peptide **18a** in varying solvent systems as a function of time. The yield is calculated from the integration of the product peak in the total HPLC chromatogram (214 nm): (squares) MeOH/1% AcOH; (circles) MeOH; (triangles) Dioxane; (diamonds) Dioxane/ 10% hydrazine;

useful for the synthesis of cyclic peptides, Bourne et al found better cyclization yields between a sterically encumbered Ile, Pro cyclization site.[16]

We wanted to overcome the extensive oligomerization that troubles the cyclization of small cyclic peptides, by developing a versatile ring closure/ring contraction strategy. We initially selected the ethanethiol auxiliary for this purpose and had found that, for larger peptides with unhindered cyclization sites (Gly-Gly), ring closure and ring contraction proceeded satisfactorily. These results were in good agreement with the work of Shao et al.,[27] who reported a similar approach for the synthesis of large cyclic peptides using unhindered cyclization sites. Also Botti et al.[28,29] reported an elegant ring closure/ring contraction process that involved an O-to-N acyl transfer via a tricyclic transition state. However, for more hindered cyclization sites or for "difficult" sequences such as our model peptide, we found that the desired ring contraction does not proceed at all. Thus the simple strategy of extending the peptide sequence with a flexible ethanethiol moiety avoids oligomerization in the cyclization process, but for "difficult" sequences such as our model peptide no ring contraction occurs even after prolonged heating.

On the basis of molecular modeling investigations, we concluded that the ring contraction is impeded by a low reactivity of the alkylthioester toward secondary amines, rather than by a sterically constrained transition-state geometry as a result of the extra atoms of the auxiliary. This is further supported by the fact that in our cyclization experiments on peptide **8** no intermolecular acyl transfer (i.e., oligomerization and not requiring a bicyclic transition state) occurred upon prolonged heating of the cyclic thioester **11**. In the presence of hydroxide anions, the thioester hydrolyzed followed by oxidation of the thiol to the disulfide **12**. This suggests that ring contraction may be possible for difficult sequences when more reactive intermediate esters are formed.

We thus focused on optimizing the acyl transfer step and discovered that the more reactive nitrobenzyl auxiliaries (**2** and **3**) provide enhanced intermolecular acyl transfer rates that are very tolerant to steric bulk at the acyl transfer site.[23] When used for the synthesis of cyclic peptides, the nitro-substituted auxiliaries **2** and **3** yielded the desired N-substituted monocyclic products in 50% and 39% isolated yield, respectively. The ring closure/ring contraction process is complete in 24 h at room temperature, or by heating to 65 °C for 1 h. This is a remarkable result, considering that no monocyclic product is obtained from the same sequence without the auxiliary.

While the 5-nitro-2-hydroxybenzyl auxiliary (**2**) is not readily removed after cyclization, we observed that photolysis at 366 nm efficiently removed the 6-nitro-2-hydroxybenzyl auxiliary (**3**) for compounds **18a**−**c** and generates the target product in good yield and purity. The rate of photocleavage is highly dependent on the selected conditions. In general a basic environment significantly reduces the photolysis rate and could be used to protect the N-substituted products **18** from premature photolysis, if required. Similarly, the linear *N*-substituted precursors **10** in their neutral form appear to be more stable to photolysis presumably due to the presence of the internal secondary amine. This simplifies the ring contraction strategy as it is not necessary, at least in the studied cases, to protect these compounds from light at any stage in the process. Further, the standard laboratory light does not produce sufficient levels of UV light at 366 nm to cause significant photolysis of *N*-substituted products **18**.

Consequently, we have developed an auxiliary that can be easily introduced at the *N*-terminus of any amino acid (except Pro) and that preorganizes the peptide prior to lactamization by formation of a larger intermediate phenolic ester, thus reducing entropic loss upon ring contraction and formation of the target monocycle. This intermediate is suitably activated to undergo rapid acyl transfer, even in the presence of steric bulk at the cyclization site. The auxiliary is then readily removed by photolysis. By using this photolabile auxiliary, we obtained cyclo-[Ala-Phe-Leu-Pro-Ala] in 21% overall yield (after isolation) from a linear precursor which in absence of the auxiliary produced no target product at all.

Conclusion

Cyclic peptides or cyclic peptidomimetics have been the subject of intensive research, especially in the pharmaceutical industry. A large number of these targeted molecules remain inaccessible, despite the growing interest. In this work we have successfully introduced a novel auxiliary strategy that will significantly expand the repertoire of cyclic peptides and peptidomimetics that are available synthetically.

The development of this activated and reversible auxiliary has implications in other peptide research areas, such as native ligation and backbone substitution, where current reversible auxiliaries suffer from a rate-limiting acyl transfer step. We are investigating the use of the auxiliary in these areas in addition to the development of cyclic peptide libraries.

Experimental Section

Materials and Methods. Chlorotrityl resin (sv = 0.92 mmol/g) was purchased from PepChem (Tubingen, Germany). All Wang resins and N_α-*tert*-butoxycarbonyl-L-amino acids were peptide synthesis grade purchased from Auspep (Melbourne Australia), Novabiochem (San Diego) or Peptide Institute (Osaka, Japan). Pam (phenylacetamidomethyl) resins were purchased from Applied Biosystems (Foster City, CA). Dichloromethane, diisopropylethylamine, *N*,*N*-dimethylformamide, and trifluoroacetic acid were obtained from Auspep (Melbourne, Australia). *p*-Cresol, *p*-thiocresol, 3-nitrophenol, 5-nitro-2-hydroxybenzaldehyde, polyphosphoric acid, and hexamethylenetetramine were purchased from Aldrich or Fluka (Sydney, Australia). HPLC grade acetonitrile was purchased from BDH (Brisbane, Australia). 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU) and benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP) were purchased from Richelieu Biotech-

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nologies (Quebec, Canada). Deionized water was used throughout. Screw-cap glass peptide synthesis reaction vessels (10 mL) with sintered glass filter frit were obtained from Embell Scientific Glassware (Queensland, Australia). Argon, helium, and nitrogen (all ultrapure grade) were from BOC (*tert*-butoxycarbonyl) gases (Queensland, Australia). ^{1}H NMR and ^{13}C NMR spectra were recorded on a 300 MHz instrument, and chemical shifts are reported in parts per million (ppm) downfield from $(CH_3)_4Si$. Reversed-phase high-performance liquid chromatography was performed on a microbore (C-18, 3 μm, 0.21 cm × 5 cm) column, an analytical (C-18, 5 μm, 0.46 cm × 25 cm) column, or a preparative (C-18, 10 μm, 2.2 cm × 25 cm) column. Chromatographic separations were achieved using linear gradients of buffer B in A (A = 0.1% aqueous TFA [trifluoroacetic acid]; B = 90% CH_3CN, 10% H_2O, 0.09% TFA) at a flow rate of 0.25 mL/min (microbore), 1 mL/min (analytical) and 8 mL/min (preparative).

Mass Spectrometry. Mass spectra were acquired on a triple quadrupole mass spectrometer equipped with an Ionspray atmospheric pressure ionization source. Samples (10 μL) were injected into a moving solvent (30 μL/min; 50:50 CH_3CN/0.05% TFA) coupled directly to the ionization source via a fused silica capillary interface (50 mm 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 (100−120 mm diameter) at a potential of 80 V. Full scan mass spectra were acquired over the mass range 400−2000 Da with a scan step size of 0.1 Da. Molecular masses were derived from the observed m/z values. LC/MS runs were carried out using a linear gradient on a dual syringe pump solvent delivery system and a reversed-phase microbore (C-18, 3.5 μm, 0.21 cm × 5 cm) column at a flow rate of 150 μL/min. Samples (typically 5 μL of 1 mg/mL solution) were loaded directly on the column, and the eluent was directly connected to the mass spectrometer via a 30 cm, 75 mm i.d., fused silica capillary. The application of Turbo Ionspray (5 L/min N_2 at 500 °C) allowed the introduction of the total eluent without splitting and loss in sensitivity. Acquisition parameters were as described above.

Monte Carlo Conformational Search. Conformational searches were performed both with and without transition-state constraints on both unsubstituted tetra-alanine and auxiliary-containing tetra-alanine. The difference in energy between the low-energy constrained and unconstrained conformations was considered to be the activation energy required to bring the molecule into the transition-state geometry. Each conformational search was performed in two steps, using the AMBER* force-field and the gb/sa continuum solvent model for water. Initially 15 000 iterations of Monte Carlo were performed where any backbone bond was free to be included in the search. If a peptide bond was chosen, it was always flipped. Only 250 minimization iterations were performed per conformer, and if after 100 iterations the conformer was >100kJ/mol from the lowest-energy conformer, it was rejected. If after 250 minimization iterations the conformer was >50 kJ/mol from the lowest-energy conformer, it was rejected. In the second step, the conformers found from the Monte Carlo search were fully minimized using conjugate gradients. Identity checks against other conformers were performed to exclude duplicates.

Chiral Amino Acid Analysis. Determination of the chirality of peptides was performed according to the method of Goodlett[30] with the following modifications. Hydrolysate: samples were hydrolyzed in 6 N HCl for 24 h at 105 °C using a Pico-Tag workstation. Trace amounts of HCl were removed using a Savant speedivac. Derivatization: hydrolysates were suspended in 100 μL of 1 M $NaHCO_3$ and 10 μL of 38.7 mM 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide in acetone. Samples were then incubated at 40 °C for 60 min. The reaction was quenched with the addition of approximately 300 μL of 1 N HCl. The samples were injected directly into the HPLC with no dilution. HPLC analysis was performed on a YMC basic column (0.46 cm × 250 cm) using a gradient of 0−100% B over 45 min, with the column heated to 45 °C and with UV detection at 340 nm (buffer A, 100 mL of methanol, 50 mL of acetonitrile, 850 mL of stock buffer; buffer B, 100 mL of methanol, 600 mL of acetonitrile, 300 mL of stock bufffer; stock buffer was ammonium formate 10 mM pH 5.2).

Peptide Synthesis. All linear peptides were chemically synthesized stepwise using either Fmoc (9-fluorenylmethyloxycarbonyl) or Boc protecting groups and in situ HBTU activation protocols as previously described.[31,32] Coupling efficiencies were determined by the quantitative ninhydrin test[33] and recoupled where necessary to obtain >99.5% efficiency.

Reductive Amination. The selected auxiliary-aldehyde (0.1 M) was dissolved in MeOH/DMF (1:1) or DMF/AcOH (100:1) and added to the amino peptide resin (2 equiv). After 5 min the resin was filtered and a second portion of aldehyde (2 equiv) added. After another 5 min the resin was filtered and washed with MeOH/DMF (1:1) or DMF/$NaBH_4$ (10 equiv) in MeOH/DMF (1:3) was then added and the reaction mixture was left standing for 5 min. The resin was again filtered and washed with MeOH/DMF (1:3), DMF, MeOH/DCM (1:1) (DCM = dichloromethane) and air-dried prior to cleavage.

Cleavage. Peptides on chlorotrityl resin were cleaved using 1% TFA in DCM (10 mL/500 mg resin, 1 h at room temperature). The resin was filtered and washed with 50% buffer B (see HPLC section), the filtrates were combined, and DCM was evaporated in vacuo. The solution containing the crude product was then loaded onto a preparative Vydac column, and the products were separated using a 1.5% linear gradient from 100% A to 20% A. Fractions were subjected to MS analysis.

Peptides on Wang resin were cleaved using TFA/water (95:5) [10 mL/500 mg resin, 1 h at room temperature]. The resin was filtered and washed with neat TFA. TFA was then removed in vacuo and the residue dissolved in 50% buffer B (see HPLC section) for HPLC purification (as above).

Peptides on PAM resin were cleaved as follows: 300 mg of resin was mixed with 0.5 mL of *p*-cresol and 0.5 mL of *p*-thiocresol, 9 mL of HF was added at 0 °C, and the mixture was stirred at 0 °C for 1 h. After evaporation of the HF the crude product was precipitated and washed with ether (2 × 10 mL). The precipitate was then dissolved in 50% CH_3CN in water (0.095% TFA) for HPLC purification (as above).

***S*-(4-Methylbenzyl)-2-thioethanol (5).** 2-Bromoethanol (25.0 g, 0.20 mol) was added dropwise to 4-methylbenzylmercaptan (27.6 g, 0.20 mol) in DMF (200 mL) at 0 °C. Triethylamine (29.3 mL, 0.21 mol) was then added dropwise over a 1 h period. The solution was allowed to stir for 3 h. Solids were filtered, H_2O (400 mL) was added, and the mixture was extracted with EtOAc (3 × 150 mL). The extracts were combined and washed with H_2O (2 × 100 mL). The organic layer was dried over $MgSO_4$, and the volatiles were removed in vacuo. The product **5** was purified by distilation at 130−132 °C (4.6 mm Hg) to yield a colorless oil (26.2 g, 75%) ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 7.21 (d, J = 8.2 Hz, 2H), 7.13 (d, J = 8.2 Hz, 2H), 3.69 (s, 2H), 3.65 (t, J = 6.0 Hz, 2H), 2.63 (t, J = 6.0 Hz, 2H), 2.33 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ 137.04, 135.11, 129.49, 128.92, 60.35, 35.58, 34.54, 21.29.

Ala-Phe-Leu-Pro-Ala. Synthesized on chlorotrityl resin (0.96 mmol/g) on a 0.1 mmol scale. Yield after TFA cleavage was 23 mg (45%): ES-MS M_r 517.1, calcd for $C_{26}H_{39}N_5O_6$, 517.3 (monoisotopic).

(D)Ala-Phe-Leu-Pro-Ala. Synthesized on chlorotrityl resin (0.96 mmol/g) on a 0.1 mmol scale. Yield after TFA cleavage was 16 mg (31%): ES-MS M_r 517.1, calcd for $C_{26}H_{39}N_5O_6$, 517.3 (monoisotopic).

Phe-Leu-Pro-Ala-Ala. Synthesized on Boc-Ala-PAM resin (0.75 mmol/g) on a 0.1 mmol scale. Yield after HF cleavage was 47 mg (91%): ES-MS M_r 517.2, calcd for $C_{26}H_{39}N_5O_6$, 517.3 (monoisotopic).

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***N*-(2-Mercaptoethanyl)-Ala-Phe-Leu-Pro-Ala (8).** Ala-Phe-Leu-Pro-Ala-Wang resin was prepared starting from Fmoc-Ala-Wang resin (0.44 mmol/g) using standard Fmoc-SPPS (SPPS = solid-phase peptide synthesis) protocols.[32] To 500 mg of resin was added *o*-nitrobenzenesulfonyl chloride (300 mg) in DMF (4 mL) containing DIEA (200 μL). After 30 min, the resin was drained and washed with DMF (3 × 6 mL). The resin was mixed with a solution of *S*-(*p*-methylbenzyl)-2-mercaptoethanol (270 mg, 1.5 mmol) in DCM (5 mL). Triphenylphosphine (393 mg, 1.5 mmol) and diethylazodicarboxylate (DEAD [diethyl azodicarboxylate], 261 mg, 1.5 mmol) were premixed in DCM (5 mL), and after 1 min, the solution was added to the resin and the reaction was left for 30 min. The resin was then washed with DCM (3 × 6 mL) and DMF (3 × 6 mL) and further treated with a solution of PhSNa (200 mg, 1.5 mmol) in DMF (4 mL) for 30 min. The resin was finally washed with DMF (3 × 6 mL) and MeOH/DCM (3 × 6 mL) and air-dried. After HF cleavage HS-$(CH_2)_2$-NH-CH(CH_3)-CO-Phe-Leu-Pro-Ala-OH was separated in 22% yield (25 mg): M_r 577.1, calcd for $C_{28}H_{43}N_5O_6S$, 577.3.

***N*-(5-Nitro-2-hydroxybenzyl)-Ala-Phe-Leu-Pro-Ala (9a).** Synthesized on chlorotrityl resin (0.96 mmol/g) on a 0.2 mmol scale. Yield after TFA cleavage was 59 mg (45%): ES-MS M_r 668.2, calcd for $C_{33}H_{44}N_6O_9$, 668.3 (monoisotopic).

***N*-(6-Nitro-2-hydroxybenzyl)-Ala-Phe-Leu-Pro-Ala (10a).** Synthesized on chlorotrityl resin (0.96 mmol/g) on a 0.1 mmol scale. Yield after TFA cleavage was 22 mg (34%): ES-MS M_r 668.2, calcd for $C_{33}H_{44}N_6O_9$, 668.3 (monoisotopic).

***N*-(6-Nitro-2-hydroxybenzyl)-Phe-Leu-Pro-Ala-Ala (10c).** Synthesized on chlorotrityl resin (0.96 mmol/g) on a 0.2 mmol scale. Yield after TFA cleavage was 67 mg (51 %): ES-MS M_r 668.2, calcd for $C_{33}H_{44}N_6O_9$, 668.3(monoisotopic).

Cyclization Experiments. Cyclization of auxiliary-containing peptides **9** and **10**: 1 equiv of BOP and 2 equiv of DIEA in DMF were added to a 1 mM solution of the linear peptide in DMF and stirred for 3 h at room temperature. Ten equivalents of DIEA was then added and the solution heated at 65° for 1 h. DMF was removed in vacuo and the crude product dissolved in acetonitrile/water (1:1) and purified by RP-HPLC (reversed-phase high-performance liquid chromatography). Cyclization of other linear peptides: Cyclizations were performed using a 1 mM solution of linear peptide in DMF. Three equivalents of BOP and 5 equiv of DIEA were added, and the solution was stirred for 3 h at room temperature. Workup was as described above.

Cyclo-[*S*-$(CH_2)_2$-Ala-Phe-Leu-Pro-Ala] (11). Cyclization of HS-$(CH_2)_2$-Ala-Phe-Leu-Pro-Ala **8** (10 mg of the TFA salt, 0.014 mmol) produced the monocyclic thioester **11** (3.4 mg, 45% yield): M_r, 559.3, calcd for $C_{28}H_{41}N_5O_5S$, 559.3. The thioester was hydrolyzed using aqueous ammonium bicarbonate buffer (0.1 M, pH 8, 6h at 60 °C) to form the *C*-terminal amides and acids. Under the mild base conditions these thiol-products oxidized to the disulfides **12** which were characterized by ES-MS: [S-$(CH_2)_2$-NH-CH(CH_3)-CO-Phe-Leu-Pro-Ala-NH_2]$_2$ M_r, 1150.8, calcd for $C_{56}H_{86}N_{12}O_{10}S_2$, 1150.6; [S-$(CH_2)_2$-NH-CH($CH_3$)-CO-Phe-Leu-Pro-Ala-$NH_2$]-S-$(CH_2)_2$-NH-CH($CH_3$)-CO-Phe-Leu-Pro-Ala-OH M_r, 1151.8, calcd for $C_{56}H_{85}N_{11}O_{11}S_2$, 1151.6; [S-$(CH_2)_2$-NH-CH($CH_3$)-CO-Phe-Leu-Pro-Ala-OH]$_2$ M_r, 1152.8, calcd for $C_{56}H_{84}N_{10}O_{12}S_2$, 1152.6.

Cyclo-[Phe-Leu-Pro-Ala-Ala]. Cyclization of Phe-Leu-Pro-Ala-Ala produced the cyclo-[Phe-Leu-Pro-Ala-Ala] in 6% yield: ES-MS M_r 499.4, calcd for $C_{26}H_{37}N_5O_5$, 499.3 (monoisotopic).

Cyclo-[Phe-Leu-Pro-(D)Ala-Ala]. Cyclization of Phe-Leu-Pro-(D)-Ala-Ala produced the cyclo-[Phe-Leu-Pro-(D)Ala-Ala] in 55% yield: ES-MS M_r 499.3, calcd for $C_{26}H_{37}N_5O_5$, 499.3 (monoisotopic).

Cyclo-[*N*-(5-nitro-2-hydroxybenzyl)-Ala-Phe-Leu-Pro-Ala] (17a). Cyclization of *N*-(5-nitro-2-hydroxybenzyl)-Ala-Phe-Leu-Pro-Ala **9a** (30 mg of the TFA salt, 0.038 mmol) produced **17a** (12.5 mg, 0.019 mmol) in 51% yield: ES-MS M_r 650.2, calcd for $C_{33}H_{42}N_6O_8$, 650.3 (monoisotopic). ^{1}H NMR (500 MHz, DMSO-d_6, ppm) (DMSO = dimethylsulfoxide) δ 11.5 (s, 1H, OH), 8.40 (d, 1H, NH_{Leu}), 8.02 (dxd, 1H, *H*-ar), 7.70 (d, 1H, *H*-ar), 7.4 (d, 1H, *H*N$_{Phe}$), 7.20−7.30 (m, 5H, *H*-Phe), 6.99 (d, 1H, *H*-ar), 6.54 (d, 1H, *H*N$_{Ala}$), 5.00 (s, 1H, ArCH*h*N), 4.91 (m, 1H, α-Ala^5), 4.75 (q, 1H, α-Ala^1), 4.59 (m, 1H, α-Phe), 4.50 (m, 1H, α-Leu), 4.27 (t, 1H, α-Pro), 3.88 (d, 1H, ArCH*h*N-), 3.62 (m, 1H, δ-Pro), 3.37 (m, 1H, δ-Pro), 2.97 (m, 1H, β-Phe), 2.82 (m, 1H, β-Phe), 2.04 (m, 2H, β-Pro), 1.88 (m, 1H, γ-Pro), 1.73 (m, 1H, β-Leu), 1.65 (m, 1H, γ-Pro), 1.44 (m, 1H, γ-Leu), 1.33 (m, 1H, β-Leu), 1.24 (d, 3H, β-Ala^5), 0.91 (d, 3H, β-Ala^1), 0.85 (m, 6H, δ-Leu). ^{13}C NMR (75 MHz, DMSO-d_6, ppm) δ 172.61, 170.34, 170.07, 169.95, 169.47, 160.40, 139.73, 136.88, 129.31, 128.14, 126.50, 125.72, 124.21, 122.65, 115.00, 61.04, 56.50, 55.74, 48.70, 46.31, 44.34, 41.37, 38.28, 31.30, 24.20, 22.81, 22.68, 21.17, 18.97, 15.35.

Cyclo-[*N*-(6-nitro-2-hydroxybenzyl)-Ala-Phe-Leu-Pro-Ala] (18a). From cyclization of *N*-(6-nitro-2-hydroxybenzyl)-Ala-Phe-Leu-Pro-Ala **10a** (20 mg of the TFA salt, 0.025 mmol), **18a** (6.5 mg, 0.010 mmol) was obtained in 39% yield: ES-MS M_r 650.6, calcd for $C_{33}H_{42}N_6O_8$, 650.3 (monoisotopic). ^{13}C NMR (75 MHz, CD_3OD, ppm) δ 178.07, 176.95, 174.54, 174.32, 173.72, 159.11, 153.19, 140.41, 131.99, 129.96, 129.54, 127.57, 121.18, 116.57, 62.75, 60.67, 58.55, 54.05, 51.15, 44.54, 43.41, 34.85, 33.67, 25.03, 24.13, 22.30, 21.31, 15.49, 13.89.

Cyclo-[*N*-(6-nitro-2-hydroxybenzyl)-Phe-Leu-Pro-Ala-Ala] (18c). From cyclization of the *N*-(6-nitro-2-hydroxybenzyl)-Phe-Leu-Pro-Ala-Ala (20 mg of the TFA salt, 0.025 mmol), **18a** (7.3 mg, 0.011 mmol) was obtained in 44% yield: ES-MS M_r 650.2, calcd for $C_{33}H_{42}N_6O_8$, 650.3 (monoisotopic). ^{13}C NMR (75 MHz, DMSO-*d*6, ppm) δ 171.43, 171.00, 169.46, 167.56, 156.65, 138.43, 129.24, 129.05, 128.32, 128.18, 126.08, 119.50, 115.87, 114.60, 62.18, 60.69, 51.07, 49.38, 46.57, 45.46, 41.54,38.17, 33.65, 31.43, 24.37, 22.73, 22.32, 21.06, 17.87, 16.92.

Cyclo-[Ala-Phe-Leu-Pro-Ala] (19a). (a) Cyclo-[*N*-(6-nitro-2-hydroxybenzyl)-Ala-Phe-Leu-Pro-Ala] (1 mM MeOH) was purged with nitrogen for 30 min and then photolyzed with a standard laboratory UV lamp (366 nm, 0.25A) for 3 h. The MeOH was evaporated, the residue was dissolved in 50% buffer B, and the solution was loaded directly onto a Vydac C18 column (preparative) for HPLC purification. Cyclo-[Ala-Phe-Leu-Pro-Ala] was isolated in 52% yield. The product was coeluted with a independently synthesized sample: ES-MS M_r 499.4, calcd for $C_{26}H_{37}N_5O_5$, 499.3 (monoisotopic). (b) Photolysis of purified cyclo-[*N*-(6-nitro-2-hydroxybenzyl)-Phe-Leu-Pro-Ala-Ala] was perfomed as described above. Cyclo-[Phe-Leu-Pro-Ala-Ala] was isolated in 28% yield. The product coeluted with a independently synthesized sample: ES-MS M_r 499.1, calcd for $C_{26}H_{37}N_5O_5$, 499.3 (monoisotopic).

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