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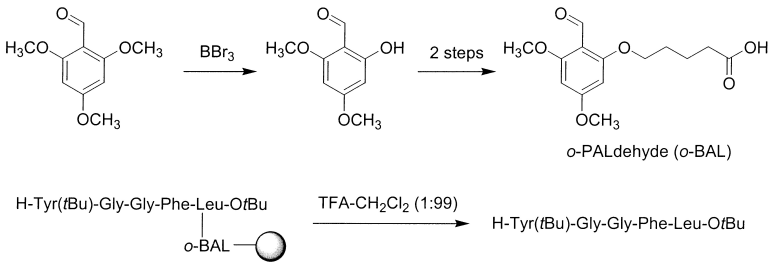

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The Ortho Backbone Amide Linker (*o*-BAL) Is an Easily Prepared and Highly Acid-Labile Handle for Solid-Phase Synthesis

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The tris(alkoxy)benzyl backbone amide linker (BAL) has found widespread application in solid-phase synthesis. The key intermediate for preparation of para BAL (*p*-BAL) is 2,6-dimethoxy-4-hydroxybenzaldehyde; several reports on its synthesis have appeared. However, the ortho analogue of the handle (*o*-BAL) has successfully been used by us for the synthesis of C-terminal-modified peptides, oligosaccharides, and substituted anilines. Here, we present a new and convenient synthesis of the key intermediate for *o*-BAL, 4,6-dimethoxy-2-hydroxybenzaldehyde, by a highly regioselective demethylation with BBr_3, followed by purification through steam distillation. Cleavage studies of Leu-enkephalin anchored to either *o*-BAL or *p*-BAL handles revealed that both handles were surprisingly acid-labile and released the peptide with dilute TFA (5% and even 1% TFA in CH_2Cl_2). This useful property allowed the synthesis of fully protected Leu-enkephalin. The very convenient synthesis of 4,6-dimethoxy-2-hydroxybenzaldehyde combined with the benign properties of the *o*-BAL handle may make it the preferred regioisomer.

Introduction

Handles (linkers) with an aldehyde functionality that allows the anchoring of substrates by convenient reductive amination have become, since their first report in the mid-1990s, widely used tools in solid-phase synthesis.[1−3] With this approach a growing peptide chain can be anchored through a backbone amide, thus giving easy access to C-terminal-modified and cyclic peptides.[3,4] This backbone amide linker (BAL)[5] concept was first implemented in a tris(alkoxy)benzyl system, which allowed release of final products by treatment with concentrated trifluoroacetic acid (TFA). Since then, BAL type handles with mono-,[6] di(alkoxy)-,[7] and alkoxyhydroxybenzyl[8] and indole[9] structures have been reported. BAL type handles have also been applied to the synthesis of small organic amides, anilines, 1,4-benzodiazepine-2,5-diones, 2,9-substituted purines, hydroxamic acids, and oligosaccharides.[3] Ley and co-workers have very recently reported a ^{13}C-labeled BAL handle to facilitate gel-phase NMR monitoring of solid-phase reactions.[10]

Most commonly, a tris(alkoxy)benzyl BAL approach commences with anchoring of 5-(4-formyl-3,5-dimethoxyphenoxy)pentanoic acid (*p*-PALdehyde), **1**, or 4-(4-formyl-3,5-dimethoxyphenoxy)butanoic acid, **2**,[11] to a solid support.[12] However, mixtures of the ortho and para aldehyde handle have also been used,[4] with the implicit assumption that an ortho analogue, e.g., 5-(2-formyl-3,5-dimethoxyphenoxy)pentanoic acid (*o*-PALdehyde), **3**, would undergo reductive amination and eventual cleavage under conditions similar to those of the para analogue (Figure 1). Besides this, the ortho analogue has rarely been used. In work from one of our groups, *o*-BAL was used in the synthesis of long peptide aldehydes,[13] substituted anilines,[14] and oligosaccharides.[15]

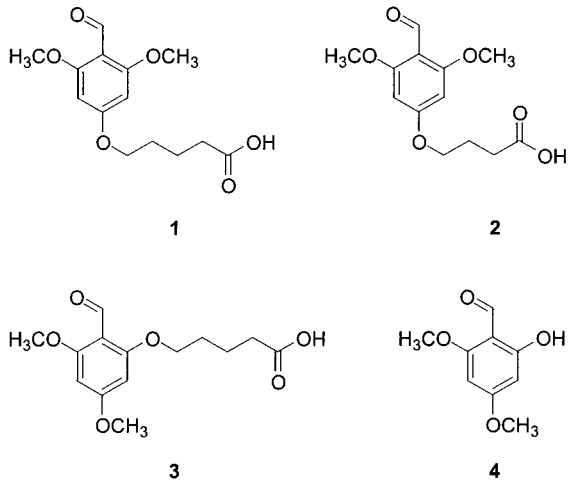

Figure 1. Structures of *p*-PALdehyde (**1**), the butanoic acid analogue (**2**), *o*-PALdehyde (**3**), and its phenol precursor (**4**).

The original procedure by Albericio and Barany for the synthesis of *p*-PALdehyde **1** consists of Vilsmeier−Haack formylation of 3,5-dimethoxyphenol, followed by selective crystallization of the para regioisomer.[16] Convenient alkylation of the phenol with ethyl 5-bromopentanoate followed by ester hydrolysis completed the synthesis. The critical Vilsmeier−Haack formylation affords a mixture of mono- and diformylated compounds, and workup can be difficult and laborious. This problem prompted Landi and Ramig to

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develop a regioselective metalation/formylation route to 2,6-dimethoxy-4-hydroxybenzaldehyde.[17] Very recently, a modification of Albericio and Barany's procedure was reported, in which trituration with $CHCl_3$ simplified workup after the formylation.[18]

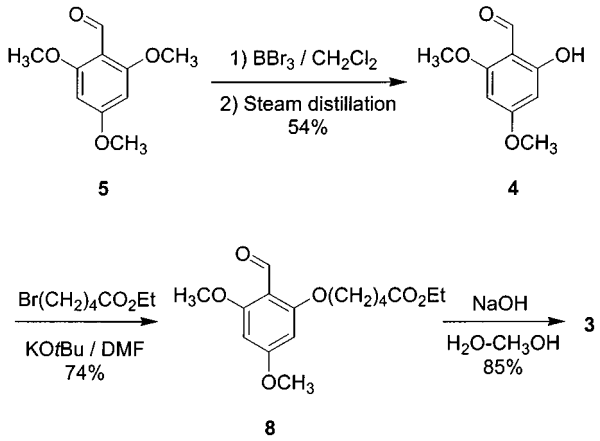
Here, we report the efficient synthesis of 4,6-dimethoxy-2-hydroxybenzaldehyde, **4**, from 2,4,6-trimethoxybenzaldehyde, **5**, by a chelation-controlled, highly regioselective demethylation reaction. Furthermore, we report explicit cleavage studies of *o*- vs *p*-BAL handles, which reveal a significantly higher acid lability than previously assumed. These findings may establish the ortho handle as the preferred regioisomer.

Results and Discussion

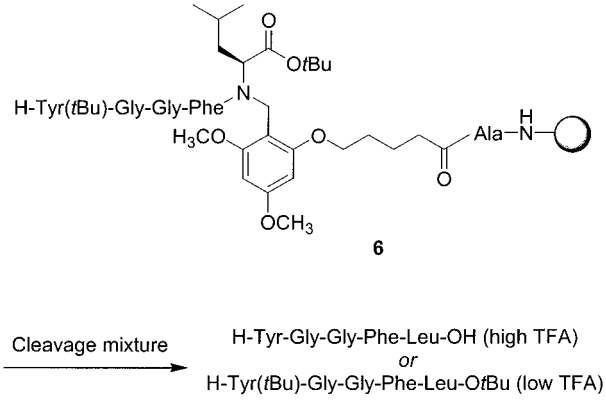
Preparation of *o*-PALdehyde. 2,4,6-Trimethoxybenzaldehyde, **5**, is a commercially available and inexpensive[19] starting material for preparation of the key compound 4,6-dimethoxy-2-hydroxybenzaldehyde, **4**. Several reports have described the chelation-controlled, regioselective demethylation of phenolic ethers ortho to a carbonyl. This requires oxophilic Lewis acids such as BCl_3,[20] BBr_3,[21] MgI_2-etherate,[22] $AlCl_3$,[23] $AlBr_3$,[24] or $CeCl_3 \cdot 7H_2O$ in the presence of NaI.[25] In contrast, demethylation by nucleophiles, e.g., thiolates[26] or *N*-methylaniline/NaH,[27] will cause random demethylation. Few reports on the demethylation of aldehyde **5** to give phenol **4** have appeared in the literature; of these, the $AlBr_3$ procedure[24] is only briefly described with no reported yield, and the $CeCl_3$/NaI procedure[25] is reported to result in partial cleavage of both ortho methoxy substituents.

Several approaches were investigated in order to achieve regioselective ortho monodemethylation af **5**. The use of 3 equiv of $AlCl_3$, following the procedure by Langmuir and co-workers,[23] gave product formation according to TLC, but workup of the reaction was troublesome because of formation of strong emulsions. Attempted demethylation of **5** with MgI_2-etherate[22] gave incomplete conversion, even when using 3 equiv of MgI_2-etherate together with prolonged reflux in toluene. However, with 1 equiv of BBr_3, complete and clean formation of the ortho monodemethylated benzaldehyde **4** was observed with short reaction times. The BBr_3 was added either neat at low temperatures (−60 °C) or as a 1 M solution in CH_2Cl_2 at higher temperatures (∼4 °C). Initially, purification was performed by an aqueous extraction procedure, similar to the procedure by Albericio and Barany. However, the ortho relationship of the phenol and the aldehyde moieties makes **4** amenable to purification by steam distillation. Addition of water to the reaction mixture followed by heating indeed facilitated steam distillation, without the need for external steam, to give **4** in a yield of 54% (Scheme 1).[28] The product was obtained as off-white crystals, which compares favorably to the classical procedure, which often gave more or less orange material. Upon standing, compound **4** gradually turned light-orange. The subsequent etherification of the ortho hydroxy group proceeded smoothly with ethyl 5-bromopentanoate in the presence of KO*t*Bu in DMF at elevated temperatures, similar to procedures described by Albericio and Barany, giving the ether compound in typically 65−75% yield. Saponification of the ethyl ester was performed with LiOH in THF−H_2O (1:1) or with 2 M aqueous NaOH−CH_3OH (1:1).[16] Good yields of typically 85% were obtained from both methods.

Scheme 1. Preparation of *o*-PALdehyde **3** via Chelation-Controlled Regioselective Demethylation of 2,4,6-Trimethoxybenzaldehyde **5**



Scheme 2. Acidolytic Cleavage of Leu-Enkephalin Anchored to *o*-Bal (**6**) and *p*-Bal (**7**)



Cleavage Studies. BAL anchored peptides have typically been released by treatment with TFA−H_2O (19:1). However, for many applications, milder cleavage conditions are desirable. To establish the mildest conditions for efficient release, Leu-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) was synthesized on both *o*- and *p*-BAL (Scheme 2). The syntheses commenced with coupling of an internal reference amino acid[29] (IRAA), Fmoc-Ala-OH, to aminomethylated polystyrene resin (0.40 mmol/g). After deprotection, the resin was split into two portions with *p*-PALdehyde **1** being coupled to one portion and *o*-PALdehyde **3** to the other. Both resins were then subjected to the same reactions, starting with reductive amination with H-Leu-O*t*Bu·HCl. The second amino acid, Fmoc-Phe-OH, was coupled to the resulting secondary amine as the preformed symmetrical anhydride, while chain elongation with the remaining residues followed a standard HBTU coupling protocol. In Fmoc deprotection of the terminal Tyr residue, the loading of the pentapeptide was quantified from the absorption of the dibenzofulvene (DBF)−piperidine adduct. The peptide synthesized on *o*-BAL, **6**, yielded 0.19 mmol/g, compared to 0.21 mmol/g for the peptide assembled on *p*-BAL, **7**. Similar, though slightly lower, loadings were calculated after hydrolysis and amino

Table 1. Cleavage Yield of Leu-Enkephalin Synthesized on *o*- and *p*-BAL Handles

		o-BAL cleavage yield, %		*p*-BAL cleavage yield, %	
entry	cleavage condition	HPLC[a]	AAA[b]	HPLC	AAA
1	reagent B,[c] 2 h	95	91	95	93
2	reagent K,[d] 2 h	91	90	95	93
3	TFA−H_2O (19:1), 2 h	91	87	94	89
4	TFA−H_2O (19:1), 15 min	82	80	81	82
5	TFA−CH_2Cl_2 (1:1), 15 min	84	82	86	83
6	TFA−CH_2Cl_2 (1:19), 15 min	75	71	69	70
7	TFA−CH_2Cl_2 (1:99), 2 h	74	70	62	64
8	TFA−CH_2Cl_2 (1:99), 15 min	28	22	20	20
9	AcOH−H_2O (19:1), 2 h	0	0	0	0
10	HFIP−CH_2Cl_2 (1:4), 2 h	0	0	0	0

[a] Yield determined by HPLC quantification of cleaved Leu-enkephalin. [b] Yield determined from the ratio of Leu to Ala (IRAA) in AAA of hydrolyzed resin after cleavage. [c] TFA−PhOH−H_2O−*i*Pr_3SiH (88:5:5:2). [d] TFA−PhOH−H_2O−$PhSCH_3$−EDT (82.5:5:5:5:2.5).

acid analysis (AAA) of the resins: 0.17 and 0.19 mmol/g for **6** and **7**, respectively.[30]

The conditions used to cleave peptidyl resins **6** and **7** are listed in Table 1. Following cleavage, the peptide solution was concentrated and the residue was dissolved in H_2O−CH_3CN and directly analyzed by HPLC. Workup with diethyl ether precipitation in this case resulted in significantly reduced yields. In all cases the desired crude peptide was obtained in more than 95% purity from both handles. Surprisingly, the peptide was cleaved efficiently from the handles by dilute TFA (TFA−CH_2Cl_2, 1:19, entry 6) in only 15 min, while very dilute TFA (TFA−CH_2Cl_2, 1:99, entries 7 and 8) released the peptide in 2 h. The very high acid lability of the *o*-BAL handle was further demonstrated in an experiment in which treatment with TFA−CH_2Cl_2 (1:99) for 15 min, followed by neutralization with *N*-ethyldiisopropylamine (DIEA), gave the *fully protected* crude peptide H-Tyr(*t*Bu)-Gly-Gly-Phe-Leu-O*t*Bu in 90% purity. When this cleavage mixture was not neutralized prior to evaporation, significant removal of *t*Bu groups was observed.

Cleavage *yields* were calculated from the HPLC−UV integral at the Tyr absorption maximum. As an orthogonal method, these yields were also obtained from the amount of residual peptide on the resin compared to the IRAA in AAA, following hydrolysis of the cleaved resin. In all cases the two results were in good agreement (Table 1). Whereas TFA−CH_2Cl_2 (1:99) resulted in significant cleavage (entry 7), even milder conditions, using cleavage mixtures not containing TFA, did not result in observable peptide release (entries 9 and 10). The most effective cleavage mixture was reagent B (entry 1), giving yields up to 95%. This is in agreement with the reported superiority of reagent B among a number of cleavage reagents, in terms of yield as well as purity, in cleavage from a PAL handle.[31] From a comparison of the yields from peptidyl resins **6** and **7**, very similar results were obtained under high-acid conditions (entry 1−5), whereas under milder conditions (entries 6−8) the *o*-BAL resin **6** consistently gave slightly higher cleavage yields compared to *p*-BAL resin **7**.

The high acid lability of the BAL linkage deserves a comment. Albericio and Barany have reported that the PAL handle, which anchors the peptide as the C-terminal amide, required concentrated TFA for its release.[32] Xanthenyl-based handles, e.g., XAL, allow the release of C-terminal peptide amides under milder conditions (1−5% TFA in CH_2Cl_2), but they appear more difficult to handle.[33] The higher acid lability of peptidyl-BAL linkages, compared to peptidyl-PAL, could be due to larger steric relief in the cleavage process (ground-state destabilization due to steric congestion) of an N-substituted amide vs a nonsubstituted amide.

Conclusion

A short and efficient synthetic route to *o*-PALdehyde, the aldehyde precursor to the *o*-BAL handle, was developed. A key step in the synthesis was the highly regioselective monodemethylation of an ortho position on 2,4,6-trimethoxybenzaldehyde, **5**. BBr_3, either neat or as a 1 M solution in CH_2Cl_2, was found to be the most efficient reagent. Workup was facilitated by a clean and efficient steam distillation procedure, which gave the product in high purity. Studies of the acidolytic release of Leu-enkephalin from *o*- and *p*-BAL handles showed that with concentrated TFA both handles cleaved equally well, with reagent B as the preferred cleavage cocktail. Surprisingly, both handles cleaved with very dilute TFA (1% TFA in CH_2Cl_2), where *o*-BAL gave slightly better yields.

Experimental Section

General Procedures. All solvents were distilled and/or stored over 3 or 4 Å molecular sieves as appropriate. 2,4,6-Trimethoxybenzaldehyde, ethyl 5-bromopentanoate, and BBr_3 (neat or 1 M solution in CH_2Cl_2) were purchased from Aldrich. *p*-PALdehyde **1** was prepared according to a previously published procedure[16] from commercial 2,6-dimethoxy-4-hydroxybenzaldehyde (Aldrich). TLC was performed on Merck silica gel 60 F_{254} plates, and spots were visualized by UV light at 254 nm. Melting points were measured on a Storm P. apparatus and are uncorrected. Ms. Karin Linthoe, University of Copenhagen, performed the elemental analyses.

Solid-phase synthesis was performed manually in polypropylene syringes equipped with polyethylene filters. Peptide couplings included a 5 min preactivating period, while the completeness of these reactions was assessed with ninhydrin tests. Aminomethylated polystyrene resin (0.40 mmol/g, 200−400 mesh) and amino acids were purchased from Novabiochem. HBTU and HOBt were purchased from Quantum Richelieu. Leu-enkephalin was purchased from Sigma. HPLC analyses were carried out on a Waters system (model 600 control unit, model 996 PDA detector, model

717 Plus autosampler, Millenium32 control software). For analysis of peptides a Waters Symmetry 300 C18 5 μm 3.9 × 150 mm column was used, running a 0.75 mL/min linear gradient from 10% buffer B to 60% buffer B over 30 min (buffer A, 0.1% TFA in H_2O; buffer B, 0.1% TFA in CH_3CN). AAA was performed with Waters Pico-Tag in duplicate, after hydrolyzing resin samples for 15 h at 130 °C with concentrated HCl−propionic acid (1:1) in the presence of phenol. ESI MS was performed on a Micromass LCT mass spectrometer.

4,6-Dimethoxy-2-hydroxybenzaldehyde, 4 (Procedure 1). 2,4,6-Trimethoxybenzaldehyde, **5** (10 g, 51 mmol) was dissolved in dry CH_2Cl_2 (100 mL) under argon. The mixture was cooled on an ice bath, and BBr_3 (55 mL, 1 M solution in CH_2Cl_2, 55 mmol) was added in 18 mL portions to the cold mixture, giving a deep-red solution. After 15 min, the ice bath was removed and the mixture was stirred for 2 h at room temperature. TLC (EtOAc) showed complete conversion. Water (200 mL) was added carefully (heat evolution), the flask was fitted with a distillation tube (Liebig's condenser, 29 mm internal diameter), and the mixture was heated gently to remove residual CH_2Cl_2. After addition of water (400 mL), the product was purified by steam distillation from the boiling aqueous solution. The procedure was repeated twice over a total of 8 h. The resulting aqueous product suspension was extracted with EtOAc (3 × 300 mL). The combined organic phases were dried (Na_2SO_4) and evaporated in vacuo to yield 5.0 g (54%) of the title compound as off-white crystals. Anal. Calcd for $C_9H_{10}O_4$: C, 59.34; H, 5.53. Found: C, 59.27; H, 5.44. Mp 70−71 °C (lit.[24] 70.5 °C).

4,6-Dimethoxy-2-hydroxybenzaldehyde, 4 (Procedure 2). 2,4,6-Trimethoxybenzaldehyde, **5** (5.0 g, 25 mmol) was dissolved in dry CH_2Cl_2 (50 mL) under argon. The mixture was cooled to −60 °C on an acetone−dry ice bath, and neat BBr_3 (3 mL, 27 mmol) was added slowly to the cold mixture, giving a deep-red solution. The cooling was removed, and the mixture was stirred for 2 h at room temperature. TLC (EtOAc) showed complete conversion. The mixture was cooled on ice, 4 M aqueous NaOH (34 mL) was added, and the mixture was stirred overnight at room temperature. Concentrated aqueous HCl (23 mL) was added to neutralize the mixture followed by extraction with EtOAc (2 × 100 mL). The organic fraction together with emulsion was separated from the aqueous layer and evaporated in vacuo. Remaining water was removed by decantation, and the residue was dissolved in CH_2Cl_2 and run through a short silica plug to remove colored byproducts to yield 3.6 g (79%) of the title compound as off-white crystals. Anal. Found: C, 59.32; H, 5.54. Mp 70−71 °C.

Ethyl 5-(2-Formyl-3,5-dimethoxyphenoxy)pentanoate, 8. KO*t*Bu (3.7 g, 33 mmol) was added to a suspension of **4** (5.4 g, 30 mmol) in dry DMF (20 mL), giving a deep-orange suspension. Ethyl 5-bromopentanoate (5.2 mL, 33 mmol) was added, and the mixture was stirred for 16 h at 60 °C. DMF was removed in vacuo (oil pump), and the residue was dissolved in CH_2Cl_2 (100 mL). The orange solution was washed with 10% aqueous Na_2CO_3 (2 × 100 mL), dried (Na_2SO_4), and eluted through a short silica plug to remove colored impurities. CH_2Cl_2 was removed in vacuo, and the product was crystallized from Et_2O−hexane to yield 6.9 g (74%) of the title compound as light-yellow crystals. Anal. Calcd for $C_{16}H_{22}O_6$: C, 61.92; H, 7.15. Found: C, 61.83; H, 7.19. Mp 44−45 °C.

5-(2-Formyl-3,5-dimethoxyphenoxy)pentanoic Acid (*o*-PALdehyde), 3. Saponification of **8**, following a literature procedure,[16] gave the title compound in 85% yield. Anal. Calcd for $C_{14}H_{18}O_6$: C, 59.57; H, 6.43. Found: C, 59.60; H, 6.42. Mp 102−105 °C (lit.[16] 103−104 °C).

Peptide Synthesis. Aminomethylated polystyrene resin (0.70 g, 0.40 mmol/g) was washed with DMF (3 × 10 mL) and CH_2Cl_2 (3 × 10 mL). Fmoc-Ala-OH (174 mg, 0.56 mmol), HBTU (212 mg, 0.56 mmol), HOBt (86 mg, 0.56 mmol), and DIEA (192 μL, 1.12 mmol) were dissolved in DMF (6 mL) and added to the resin. After 2 h, the resin was washed with DMF (5 × 10 mL) and CH_2Cl_2 (5 × 10 mL) and dried in vacuo. Next, the resin was split into two equal portions, which were both treated with piperidine−DMF (1:4, 2 × 5 mL, 3 + 20 min) and washed with DMF (5 × 5 mL). In two parallel reactions, a solution of *p*-PALdehyde **1** (79 mg, 0.28 mmol), HBTU (106 mg, 0.28 mmol), HOBt (43 mg, 0.28 mmol), and DIEA (96 μL, 0.56 mmol) in DMF (3 mL) was added to one resin portion, while an identical mixture with *o*-PALdehyde **3** substituted for **1** was added to the other resin portion. After 2 h, both portions were washed with DMF (5 × 5 mL) and CH_2Cl_2 (5 × 5 mL) and dried in vacuo. All subsequent steps were performed identically on both resin portions. Quantities refer to one resin portion. H-Leu-O*t*Bu·HCl (313 mg, 1.4 mmol) and $NaBH_3CN$ (88 mg, 1.4 mmol) were dissolved in DMF (3 mL) and transferred to the resins. After 1 h, the resins were washed with DMF (5 × 5 mL) and CH_2Cl_2 (5 × 5 mL). Fmoc-Phe-OH (542 mg, 1.4 mmol) and DIPCDI (108 μL, 0.7 mmol) were left to react in CH_2Cl_2−DMF (9:1, 3 mL) over 15 min. The slurry was then transferred to the resins with CH_2Cl_2 (3 mL). After 2 h, the resins were washed with DMF (5 × 5 mL) and CH_2Cl_2 (5 × 5 mL). The coupling and washing procedure was repeated twice. Next, treatment with piperidine−DMF (1:4, 2 × 5 mL, 3 + 20 min) was followed by washes with DMF (5 × 5 mL). The three final residues, Gly, Gly, and Tyr, were attached as Fmoc-Gly-OH (0.56 mmol) or Fmoc-Tyr(*t*Bu)-OH (0.56 mmol) in DMF (3 mL) with HBTU (0.56 mmol), HOBt (0.56 mmol), and DIEA (1.12 mmol) over 2 h. Following washes with DMF (5 × 5 mL), the resins were treated with piperidine−DMF (1:4, 2 × 5 mL, 3 + 20 min). From the UV absorption of the piperidine−DBF adduct ($\epsilon_{290\ nm}$ = 5800 M^{-1} cm^{-1}) loadings of 0.19 mmol/g (**6**) and 0.21 mmol/g (**7**) were found for the final deprotection. Upon completion of the syntheses, resin hydrolysis and AAA gave the following compositions and loadings: $Tyr_{1.00}Gly_{1.97}Phe_{0.90}Leu_{1.15}Ala_{1.49}$ with 0.17 mmol/g Leu loading (**6**) and $Tyr_{1.00}Gly_{2.02}Phe_{0.94}Leu_{1.18}Ala_{1.46}$ with 0.19 mmol/g Leu loading (**7**).

Cleavage Studies. Samples (10−15 mg) of **6** and **7** were cleaved simultaneously using the cleavage mixtures (1 mL) listed in Table 1. After the desired cleavage time, the resins were drained and washed with an additional amount of cleavage mixture (5 × 1 mL). The combined cleavage

mixture and washings were then concentrated to dryness, dissolved in CH_3CN-H_2O (1:1, 1 mL), and analyzed by HPLC. When cleaved with TFA−CH_2Cl_2 (1:1) or less acidic mixtures, the product was deprotected with neat TFA (2 mL), 15 min, before HPLC analysis. The peptide concentration was determined from the integral of the 275 nm absorption peak, using a standard curve obtained with commercial Leu-enkephalin. This concentration was converted to a cleavage yield using the peptide loading obtained from AAA. Next, the cleaved resin was hydrolyzed and subjected to AAA. The cleavage yield was calculated by comparing the Leu/Ala ratio with that of noncleaved resin **6** or **7**. Disregarding the scavengers present in reagents B and K, all cleavages (with subsequent TFA treatment) produced HPLC chromatograms with a single peak at $t_R = 16.4$ min (>95% purity). This product coeluted with commercial Leu-enkephalin. ESI MS Calcd for $C_{28}H_{32}N_5O_7$: 555.27. Found: *m*/*z* 556.28 $[M + H]^+$, 578.30 $[M + Na]^+$, 594.23 $[M + K]^+$. In a cleavage of **6** with TFA−CH_2Cl_2 (1:99, 1 mL), the mixture was neutralized with DIEA−CH_2Cl_2 (1:99, 5 × 1 mL) after 15 min. HPLC analysis gave one major peak at $t_R = 28.8$ min (90%) with impurities at $t_R = 23.4$ min (3%) and $t_R = 24.5$ min (5%). The main product was identified as di-*t*Bu-protected Leu-enkephalin. ESI MS Calcd for $C_{36}H_{53}N_5O_7$: 667.39. Found: *m*/*z* 668.43 $[M + H]^+$, 690.39 $[M + Na]^+$, 706.36 $[M + K]^+$. The impurities were found to be the two possible mono-*t*Bu-protected Leu-enkephalins. ESI MS Calcd for $C_{32}H_{45}N_5O_7$: 611.33. Found: *m*/*z* 612.34 $[M + H]^+$, 650.30 $[M + K]^+$. After TFA treatment of the protected peptide, the cleavage yield was calculated to be 28%, thereby reproducing the result from Table 1 (entry 8).

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