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Solid-Phase Synthesis of Phenylalanine Containing Peptides Using a Traceless Triazene Linker

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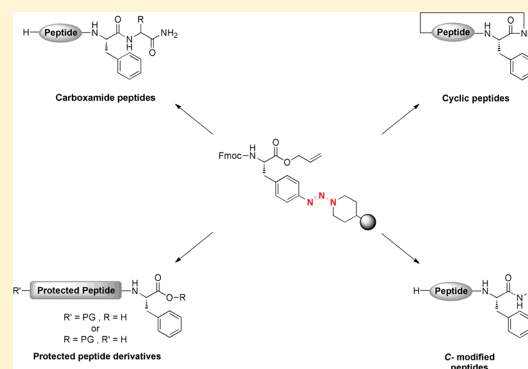
Supporting Information

ABSTRACT: The use of a triazene function to anchor phenylalanine to a polymeric support through its side chain is reported. To prove the usefulness of this strategy in solid-phase peptide synthesis, several bioactive peptides have been prepared including cyclic, *C*-modified, and protected peptides. The triazene linkage is formed by coupling the diazonium salt of Fmoc-Phe(pNH$_2$)-OAllyl to a MBHA-polystyrene resin previously functionalized with isonipecotic acid (90%). Further assembly of the peptide chain, cleavage from the resin using 2−5% TFA in DCM, and reduction of the resulting diazonium salt of the peptide with $FeSO_4 \cdot 7H_2O$ in DMF afforded the desired products in high purities (73−94%).



Peptide research on drug design and drug discovery is one of the most promising fields in the development of new drugs. However, linear peptides are generally not suitable for use as drugs because of their poor bioavailability. For this reason, much attention is being paid to the search for molecules with potential therapeutic properties together with improved structural and enzymatic stability. In this sense, cyclic peptides, either naturally occurring or synthetically constructed, and *C*-modified peptides have proven to be excellent molecular scaffolds for design purposes.[1] In this field of research, phenylalanine has become an important pharmacophore in SAR studies because of the key role that plays eliciting intrinsic activity in compounds that contain this amino acid.[2] Thus, the nonpolar nature and steric bulkiness of its side chain is responsible for crucial hydrophobic interactions in molecular recognition processes.[3] Moreover, a large number of natural peptides covering a wide range of biological properties contain at least one phenylalanine residue[1] and are, therefore, potential targets for the design of new drugs.

From a synthetic point of view, connection of the amino acid to the resin via its side chain as the starting building block is one of the most suitable approaches to the synthesis of cyclic[4] and *C*-modified[5] peptides because both extremes of the peptide chain are left free for further chemical transformations. As a part of our ongoing studies devoted to explore novel synthetic strategies based on side-chain anchoring of aromatic amino acids to solid supports,[6] we have developed a new synthetic strategy for the side-chain anchoring of phenylalanine to a polymeric support through a triazene linkage. The potential of this approach has been proven by the synthesis of some biologically active peptide derivatives.

In general, side-chain anchoring solid-phase strategies have taken advantage of Fmoc chemistry because they can be easily adapted to protection schemes such as Fmoc/^{t}Bu/Allyl,[7] and most of the proteinogenic trifunctional amino acids have been used in such strategies as starting building blocks with this purpose.[4,5] However, scarce literature can be found about phenylalanine because of its side-chain nature. In this sense, Silverman et al. have reported an elegant silicon-based linking strategy to attach the aromatic side chain of phenylalanine that has proven to be efficient in the synthesis of small peptides[8] and cyclic peptides.[9−11] Thus, peptides can be obtained in a traceless way upon cleavage from the resin under acidic conditions or halogen *ipso*-substituted phenylalanine containing peptides can be formed (Br or I) by cleavage of the peptide in the presence of suitable electrophile precursors (Br_2 or ICl).[12] Inspired by this concept, we focused our attention on the possibility of tethering phenylalanine to the polymeric support through a triazene linker. This linker group was originally introduced by Bräse et al.[13] for the solid-phase synthesis of aromatic and heteroaromatic compounds.[14] This system is easily accessible by coupling the target aromatic amine through its diazonium salt to a disubstituted amino-functionalized

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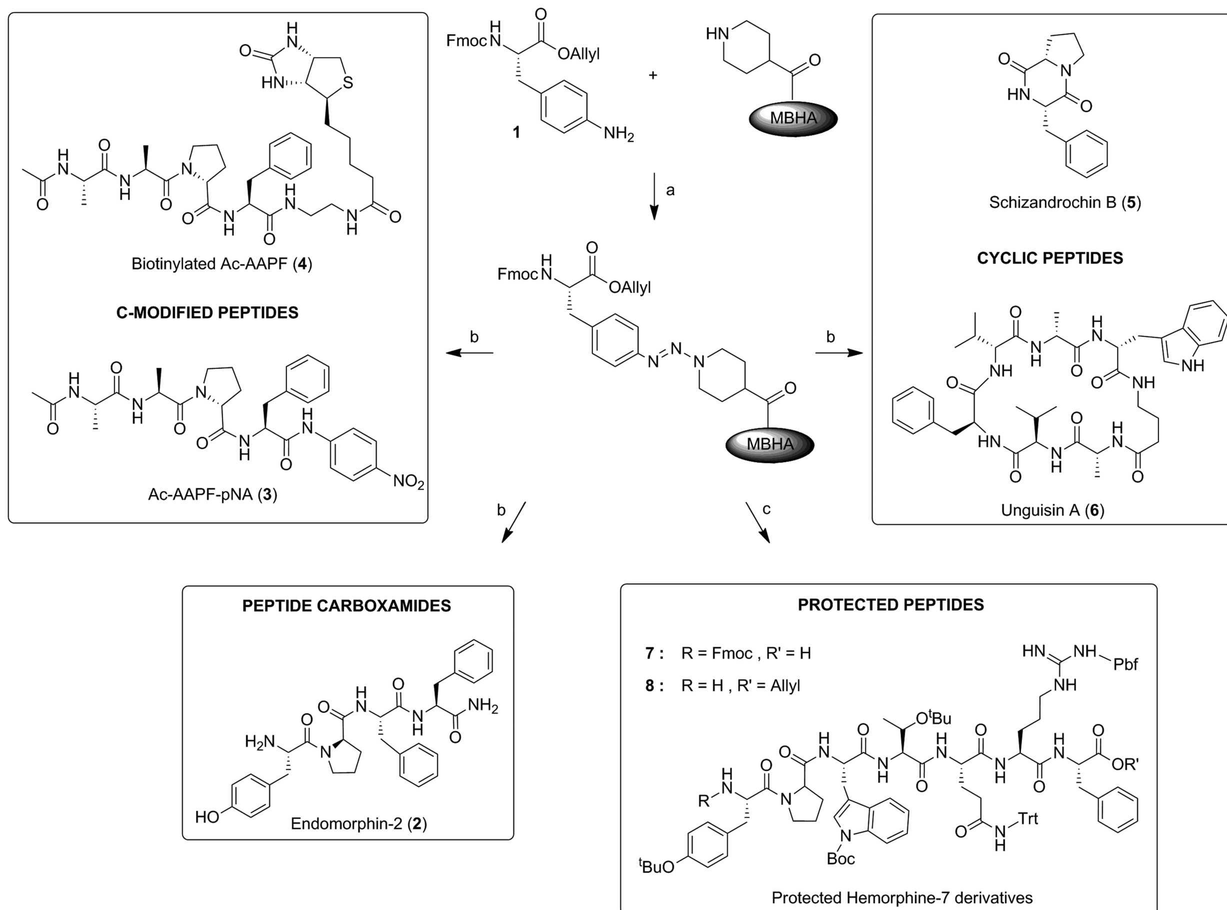
Scheme 1. Synthesis of the Peptides Reported in This Work[a]



[a]Key: (a) (i) In solution diazonium salt of **1**: BF_3Et_2/$^tBuNO_2$; DCM; −10 °C; (ii) solid-phase coupling: pyridine, DCM, rt; (b) (i) SPPS; (ii) 5% TFA in DCM, 5 min; (iii) $FcSO_4$–$7H_2O$/DMF; (c) (i) SPPS; (ii) 2% TFA in DCM, 3 × 2 min; (iii) $FeSO_4$–$7H_2O$/DMF.

polymeric support. A broad spectrum of synthetic approaches can be applied to the aromatic compound attached to the resin due to the robustness of the linker (stable toward daylight, oxygen, moisture, bases, reducing agents, or oxidizing agents). However, acidic cleavage of the triazene resin under mild conditions yields the amine resin, which can be recycled, and the modified aryldiazonium salt, which can be further reduced after the cleavage step. In this sense, the triazene linker can be envisaged as a multifunctional linker[13] because of the wide range of chemical transformations that diazonium salts can provide,[14a] which could be used in a combinatorial fashion for SAR studies.

To prove the feasibility of this synthetic approach with phenylalanine, several peptides were chosen as models (Scheme 1): endomorphin 2 (**2**),[15] an endogenous opioid tetrapeptide carboxamide with high selectivity and specificity for the μ-opioid receptor; the Ac-AAPF *p*-nitroanilide peptide (**3**),[16] a chromogenic substrate for protease activity studies; the same peptide but biotinylated at the *C*-terminal (**4**)[17] as an example of a potential molecular probe; cPhePro (**5**),[18] a DKP with antifungal and antimicrobial properties found in a wide range of organisms; and unguisin A (**6**),[19] a highly hydrophobic cyclic heptapeptide isolated from the marine fungus *Emericella unguis* and with antibacterial activity against *staphylococus aureus*.

The three-dimensional orthogonal strategy based on Fmoc/^{t}Bu/allyl groups[20] was chosen to attach phenylalanine to a solid support via triazene linkage because of the stability of this function to basic and neutral conditions. With the aim of coupling phenylalanine to the polymeric support, the *p*-amino-substituted amino acid was required (Fmoc-Phe(pNH_2)-OAllyl, **1**. This derivative was prepared following two synthetic pathways using slightly modified classical methods starting from the commercially available Fmoc-Phe(pNO_2)-OH by *C*-terminal protection with allyl bromide (98%)[21] followed by reduction of the nitro group with Zn dust in acidic conditions (69% yield).[22] We decided to use a piperazinyl-type resin[14a] to anchor **1** through its side chain. The reaction conditions to carry out this reaction were optimized from those described in the literature with a commercial piperazinylmethylpolystyrene resin, but we further moved to a MBHA-polystyrene resin modified with Boc-isonipecotic acid (DIC/HOBt in DMF) to have the possibility to control the functionalization of the polymeric support and to perform on-resin quantitative amino acid analysis by the introduction of a standard amino acid. The best coupling results were achieved (up to 91%) when the resin was treated under Ar atmosphere with 2.5 equiv of the diazonium salt of **1** (preformed with $BF_3{\cdot}Et_2O$/$^tBuNO_2$ in DCM at −10 °C, 1 h) and 30 equiv of pyridine in DCM at rt during 3 h. Final amino acid functionalization levels were determined by spectrophotometric quantification of Fmoc group.[23]

The peptides that are shown in Scheme 1 were synthesized as follows. The peptide chain of **2** was assembled from the *N*-terminal of the phenylalanine attached to the resin and was

completed with the coupling of the *C*-terminal phenylalanine carboxamide. *C*-Terminal-modified Ac-AAPF peptides **3** and **4** were synthesized using different synthetic approaches. Thus, *p*-nitroaniline was coupled to the *N*-urethane-protected phenylalanine before peptide chain elongation to avoid racemization of this amino acid under the experimental conditions used in this reaction,[24] while biotin was attached at the end of the assembly of the peptide chain. DKP **5** was prepared by coupling proline to the *C*-terminal of phenylalanine in order to perform on-resin cyclization through the primary *N*-terminal of phenylalanine. Finally, the peptide sequence of cyclopeptide **6** was grown from the *N*-terminal of phenylalanine and cyclized before cleavage from the resin.

N-Terminal peptide chain elongation was performed by anchoring stepwise N^{α}-Fmoc-protected amino acids with DIC/HOBt[25] and final acetylation with Ac_2O/DIEA in the case of **3** and **4**. Reactions were monitored until completeness using the Kaiser test for primary amines[26] or the chloranil test for proline.[27] Removal of the Fmoc protecting group was carried out with 20% of piperidine in DMF, except for **6** where 3% of the non-nucleophilic base DBU[28] in DMF was used to remove Fmoc prior to on-resin cyclization in order to avoid *C*-terminal piperidylamide formation as a byproduct during this step.[7,29] Furthermore, stronger basic conditions were necessary for **6** to carry out N^{α} deprotection after the coupling of GABA and a mixture of 10% of DBU and 10% of piperidine in DMF was required for complete removal.[30] The *C*-terminal allyl protection of phenylalanine was removed using $Pd(PPh_3)_4$ in the presence of $PhSiH_3$[31] to further attach H-Phe-NH_2 (**2**), *N*-Fmoc-diethylenediamine hydrochloride (**4**) and H-Pro-OMe·HCl (**5**) with DIC/ethyl cyanoglyoxyl-2-oxime (Oxyma)[32] following a double-coupling protocol to ensure complete reaction. In the case of peptide **3**, five treatments with *p*-nitroaniline and PyAOP/HOAt/DIEA in DMF[33] were needed to anchor the amine, as revealed by HPLC−MS analysis. This coupling system was also used to attach biotin to complete the assembly of **4**, but only one treatment was required in this particular case, as shown by the Kaiser test. On-resin cyclizations of peptides **5** and **6** were carried out following different strategies. Thus, removal of the Fmoc group from the linear dipeptide methyl ester using a mixture of 10% of DBU and 10% of piperidine in DMF took place with concomitant intramolecular cyclization to afford the desired DKP skeleton. In this sense, it is interesting to note that cyclization of the dipeptide resulting from coupling of N^{α}-Fmoc-Pro-OH to *N*-terminal of phenylalanine gave complex crudes under the same experimental conditions. Concerning **6**, after removal of *C*-terminal allyl ester and the *N*-terminal Fmoc group, the peptide backbone was head-to-tail cyclized upon activation with PyOAP/HOAt/DIEA in DMF. Both cyclization processes went to completion as monitored by the Kaiser test.

Once the linear precursors were built on the resin, peptide-resins were subjected to an acidolytic cleavage treatment. Several acids under or followed by reducing conditions have been applied to cleave small nonpeptide aromatic molecules linked to a solid support via triazene. Thus, HCl in the presence of THF,[13] H_2PO_3,[13] $HSiCl_3$,[34] or TFA followed by THF/DMF[36] have been used to obtain the traceless product. In view of these precedents, we decided to use conditions similar to those reported by Schunk et al.[35] because we believed those conditions would be more amenable to peptide chemistry protocols in terms of compatibility with amino acid side-chain functionalities. Thus, peptides **2**−**6** were cleaved from the resin with 5% TFA in DCM. Three 2 min treatments with this solution were carried out to drive the reactions to completion. In the case of peptide **2**, the tyrosine side chain remained protected under these conditions and required a treatment of 3 h with TFA/TIS/H_2O (95:2.5:2.5) in order to remove the ^{t}Bu group. However, further treatment with THF to reduce the resulting diazonium salts gave unreproducible results and complex HPLC profiles. That moved us to explore other strategies for reduction. To our surprise, the hydrodediazoniation process mediated by $FeSO_4{\cdot}7H_2O$ in the presence of DMF as hydrogen donor[36] proved to be very efficient, giving clean reaction crudes with the absence of byproducts. In general, reactions went to completion at room temperature after a few minutes, and products could be easily isolated performing a simple workup that included ion-exchange chromatography to remove any trace of iron, which was evidenced by a qualitative assay using the colorimetric reagent ammonium thiocyanate. Peptides crudes **2**−**6** were obtained with chromatographic purities of 73−94%.

In the case of peptide **2**, we decided to evaluate potential epimerization during the *C*-terminal coupling of H-Phe-NH_2.HCl to the peptide backbone despite not detecting diastereoisomers by HPLC. This reaction was performed using DIC/oxyme as coupling reagents, conditions which are reported to give rise to low degree of *C*-terminal racemization.[32] However, in order to determine the epimerization more precisely, some experiments using Ac-Phe-Phe-NH_2 (**9**) as model peptide were carried out. The dipeptide was prepared in solid phase under conditions similar to those used in the synthesis of **2**. Thus, the protected amino acid **1** was anchored to the resin, the *N*-terminal was acetylated after removing the Fmoc group, and *C*-terminal coupling of H-Phe-NH_2·HCl was carried out. The analysis of the acidolytic crude showed an 8−12% of racemization when it was compared with the corresponding standards Ac-L-Phe-L-Phe-NH_2 (**9**) and Ac-D-Phe-L-Phe-NH_2 (**10**), previously prepared on a Rink amide resin following standard solid-phase protocols. It is interesting to note that this drawback was overcome when *C*-terminal coupling was performed on an Fmoc-protected Phe residue attached to the resin, followed by Fmoc group elimination and further *N*-terminal acetylation.[24]

The cleavage of the peptide under mild acidic conditions offers the possibility of using this methodology for the synthesis of protected peptides if required for convergent strategies. In this sense, side-chain anchoring of the peptide chain allows one to obtain the protected peptide with one of the extremes unprotected if suitable orthogonal protections for the *N*-terminal or the *C*-terminal are used. This approach is exemplified by the synthesis of the side-chain protected peptide hemorphine-7, an endogenous peptide belonging to the family of opioid peptides released from hydrolyzed hemoglobin.[37] Thus, this peptide was solid-phase assembled using DIC/HOBt in DMF as *N*-terminal- or *C*-terminal-protected chains, and both were cleaved from the resin with 2% TFA in DCM (5 treatments of 2 min) to afford the side-chain-protected peptides **7** or **8**, respectively, in high yields (more than 90%) and purity (more that 93%, as determined by integration of the chromatographic peak areas).

In summary, we have developed a new versatile and efficient methodology for the solid-phase synthesis of peptides containing phenylalanine, based on the anchorage of this amino acid to the solid support through its side chain. Key aspects of this strategy include the use of Fmoc-Phe(*p*NH_2)-

OAllyl as the starting building block, coupling of this amino acid via triazene to an MBHA-polystyrene resin modified by the spacer Boc-isonipecotic acid, the use of low concentrated TFA for peptide cleavage from the resin, and mild removal of the resulting peptide diazonium salt with $FeSO_4{\cdot}7H_2O$. This strategy allows the modification of both ends of the peptide chain, as well as the synthesis of protected peptides if required for a convergent approach. Alternatively, the scope of application could be broadened by the introduction of diversity through the chemical transformation of the diazonium salt to obtain peptide analogues potentially useful for SAR studies. Investigations in this direction are currently underway.

■ EXPERIMENTAL SECTION

Materials and Methods. Solid-phase peptide synthesis was carried out in polypropylene syringes fitted with porous polystyrene frits. Reactions were performed at rt using occasional manual stirring, and the resins were washed, unless stated otherwise, with DMF (5 × 30 s), DCM (5 × 30 s), MeOH (5 × 30 s), and DCM (5 × 30 s). Couplings were monitored until completeness using the Kaiser test for primary amines[27] or the chloranil test for proline.[28] Solvents and excess of reagents were removed by filtration under reduced pressure. On-resin quantifications were carried out by hydrolysis and amino acid analysis as follows: the peptidyl resin samples (7.5−9.5 mg) were washed with DMF (5 × 30 s), DCM (5 × 30 s), and MeOH (5 × 30 s) and then dried under reduced pressure. Hydrolyses were performed using 300 μL of aqueous HCl (37% v/v) and 300 μL of propionic acid. The acidolytic crudes were dissolved in aqueous HCl (20 mM) to achieve a suitable concentration for amino acid analysis. Flash chromatography was carried out in an automated flash system. Semipreparative HPLC was carried out with a Sunfire C18, 19 × 100 mm column. XBridge C18, 4.6 × 50 mm and XBridge BEH 130 C18 columns were used for analytical HPLC−MS. NMR spectra were collected in $CDCl_3$ at 25 °C. Mass spectra were acquired with quadrupole detection and an electrospray ion source in positive-ion mode.

(*S*)-Fmoc-Phe(*p*NO$_2$)-OAllyl. (*S*)-Fmoc-Phe(*p*NO$_2$)-OH (3.07 g, 7.63 mmol) was dissolved in DMF (60 mL), and $NaHCO_3$ (2.69 g, 32.02 mmol) and allyl bromide (1.5 mL, 17.20 mmol) were added. The mixture was stirred at rt for 16 h, and then the solvent was removed. The pale yellow solid obtained was dissolved in EtOAc (60 mL) and was washed with H_2O (3 × 60 mL). The organic phase was dried, and the solvent was removed under vacuum, furnishing the product as a white solid that was used in the next step without further chromatography purification (3.52 g, 98%): mp = 143−146 °C; R_f 0.47 [^{t}BuOMe/hexanes (1:1)]; $[\alpha]^{20}_D$ = +15.6 (*c* 1, $CHCl_3$,); IR (ATR) 3329, 1749, 1687, 1516, 1338, 1263, 1213 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 (d, *J* = 8.4 Hz, 2H), 7.77 (d, *J* = 7.6 Hz, 2H), 7.56 (m, 2H), 7.41 (m, 2H), 7.30 (m, 2H), 7.22 (d, *J* = 8.3 Hz, 2H), 5.86 (m, 1H), 5.31 (m, 3H), 4.70 (m, 1H), 4.62 (d, *J* = 5.7 Hz, 2H), 4.51 (dd, J_1 = 10.9 Hz, J_2 = 6.9 Hz, 1H), 4.40 (dd, J_1 = 10.7 Hz, J_2 = 6.4 Hz, 1H), 4.19 (t, *J* = 6.4 Hz, 1H), 3.27 (dd, J_1 = 13.9 Hz, J_2 = 5.8 Hz, 1H), 3.16 (dd, J_1 = 13.8 Hz, J_2 = 5.9 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.5, 155.4, 147.2, 143.6, 143.5, 141.4, 131.0, 130.3, 127.8, 127.1, 124.9, 123.7, 120.0, 119.7, 66.8, 66.5, 54.4, 47.2, 38.1; HRMS calcd for $C_{27}H_{24}N_2O_6Na$ [M + Na]$^+$ 495.1526, found 495.1527.

(*S*)-Fmoc-Phe(*p*NH$_2$)-OAllyl (1). (*S*)-Fmoc-Phe(*p*NO$_2$)-OAllyl (1.91 g, 4.04 mmol) and Zn dust (1.29 g, 19.73 mmol) were suspended in absolute EtOH (50 mL). Glacial AcOH (50 mL) was added to the mixture, and the resulting suspension was stirred at 60 °C for 1 h. Solvent was evaporated, and the product was purified by flash chomatography using DCM/EtOAc (9:1) as eluent, affording the product as a white solid (1.24 g, 69%): mp = 124−127 °C; R_f 0.41 [DCM/EtOAc (9:1)]; $[\alpha]^{20}_D$ = +15.2 (*c* 1, $CHCl_3$); IR (ATR) 3389, 2931, 1740, 1692, 1260 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.7 Hz, 2H), 7.56 (t, *J* = 6.4 Hz, 2H), 7.39 (m, 2H), 7.30 (tt, J_1 = 4.4 Hz, J_2 = 3.7 Hz, 2H), 6.89 (d, *J* = 8.2 Hz, 2H), 6.59 (d, *J* = 8.3 Hz, 2H), 5.89 (m, 1H), 5.30 (m, 3H), 4.61 (m, 3H), 4.41 (dd, J_1 = 10.5 Hz, J_2 = 7.2 Hz, 1H), 4.32 (dd, J_1 = 10.7 Hz, J_2 = 7.0 Hz, 1H), 4.20 (t, *J* = 7.2 Hz, 1H), 3.51 (bs, 2H), 3.02 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4, 155.6, 145.4, 143.8, 141.3, 131.5, 130.2, 127.7, 127.0, 125.3, 125.2, 120.0, 119.0, 115.3, 67.0, 66.0, 55.0, 47.2, 37.4; HRMS calcd for $C_{27}H_{27}N_2O_4$ [M + H]$^+$443.1965, found 443.1962.

Isonipecotic Acid Loaded MBHA Resin. MBHA resin (1.57 g, 0.63 mmol/g, 0.99 mmol) was introduced into a polypropylene syringe fitted with a porous polystyrene frit and was washed successively with DCM (10 × 30 s), TFA (40% v/v) in DCM (1 × 1 min and 2 × 10 min), DCM (5 × 30 s), DIEA (5% v/v) in DCM (5 × 2 min), DCM (5 × 30 s), and DMF (5 × 30 s). Then, Fmoc-Gly-OH (internal reference) (0.88 g, 2.97 mmol), HOBt (0.40 g, 2.97 mmol), and DIC (460 μL, 2.97 mmol) in DMF (4 mL) were added. The suspension was left for 1 h at rt with occasional manual stirring and was washed with DMF (5 × 30 s), DCM (5 × 30 s), MeOH (5 × 30 s), and DCM (5 × 30 s). Then, Fmoc aminoacyl protection was removed with 20% piperidine in DMF (1 × 1 min and 2 × 10 min) and the Boc-isonipecotic acid (0.68 g, 2.97 mmol), HOBt (0.40 g, 2.97 mmol) and DIC (460 μL, 2.97 mmol) added in DMF (4 mL). After 1 h at rt with occasional manual stirring, the mixture was washed with DMF (5 × 30 s), DCM (5 × 30 s), MeOH (5 × 30 s), and DCM (5 × 30 s). Finally, the resin was treated with 40% of TFA in DCM to remove Boc group (2 × 10 min) and washed with DCM (5 × 30 s), DIEA (5% v/v) in DCM (5 × 2 min), DCM (5 × 30 s), MeOH (5 × 30 s), DCM (5 × 30 s), and DMF (5 × 30 s).

General Procedure A for the Coupling of Phenylalanine to the Resin via Triazene. Compound **1** (1.24 g, 2.80 mmol) was dissolved under Ar in anhydrous DCM (35 mL), and the resulting solution was cooled to −10 °C, and then $BF_3{\cdot}Et_2O$ (1.80 mL, 14.20 mmol) and ^{t}BuNO$_2$ (1.85 mL, 14.00 mmol) were added. The mixture was stirred while maintaining this temperature for 1 h under Ar and was added via cannula to a preformed mixture of the piperidine linker resin (1.57 g, 0.99 mmol) and anhydrous pyr (18 mL) at −10 °C. The resulting suspension was shaken at rt for 3 h under Ar, and then after filtration, the resin was washed with DCM (5 × 30 s), MeOH (5 × 30 s), DCM (5 × 30 s), and DMF (5 × 30 s). Spectrophotometric quantification of Fmoc groups afforded an amino acid coupling of 91% yield.

Capping of Unreacted Piperidino Groups. The aminoacyl resin was washed with DMF (5 × 30 s) and treated with Ac_2O (1.9 mL, 20.03 mmol) and DIEA (3.5 mL, 20.09 mmol) in DMF (4 mL) for 30 min. Then, the mixture was washed with DMF (5 × 30 s), DCM (5 × 30 s), MeOH (5 × 30 s), and DCM (5 × 30 s).

General Procedure B: Amino Acid Coupling. To the resin were added the amino acid (3 equiv), HOBt (3 equiv), and DIC (3 equiv) in DMF (4 mL) and the mixture reacted for 1 h at rt with occasional manual stirring. Then, the resin was washed with DMF (5 × 30 s), DCM (5 × 30 s), MeOH (5 × 30 s), and DCM (5 × 30 s).

General Procedure C: Removal of the Fmoc Group. The resin was treated with piperidine (20% v/v) in DMF (8 mL) (1 × 1 min and 2 × 10 min) or DBU (3% v/v) in DMF (1 × 1 min and 2 × 10 min) in the case of the *N*-terminal Fmoc group, and then it was washed with DMF (5 × 30 s), DCM (5 × 30 s), MeOH (5 × 30 s), and DCM (5 × 30 s).

General Procedure D: Removal of Allyl Group. The resin was washed with DMF (5 × 30 s) and DCM (5 × 30 s). Then the resin was suspended in DCM and degassed by bubbling Ar for 5 min, when $Pd(PPh_3)_4$ (0.4 equiv) and $PhSiH_3$ (48 equiv) in DCM (8 mL) were added. The mixture was shaken for 30 min at rt, filtered, and washed with DCM (8 × 30 s). This treatment was carried out twice under the same conditions. After filtration, the resin was washed with DCM (8 × 30 s), a solution of sodium diethyl dithiocarbamate (5% v/v) in DMF (2 × 5 min), DMF (5 × 1 min), and DCM (5 × 30 s).

General Procedure E: Cleavage of the Peptide from the Resin and Diazonium Salt Reduction. Cleavage of the peptide from the resin was brought about by treatment with TFA in DCM (5/95 v/v) (3 × 2 min) (peptides **2**−**6**) and (2/98 v/v) (5 × 2 min) (peptides 7 and **8**), and the collected washings were evaporated under vacuum to dryness (the diazonium salt was protected from light).

Then the diazonium salt was dissolved in DMF (15 mL), and $FeSO_4 \cdot 7H_2O$ (1.5 equiv) was added. The mixture was stirred at rt for 5 min, and the solvent was removed under vacuum. The presence of iron impurities after chromatographic purifications was evaluated as follows: a drop of a solution of peptide samples in a mixture of aqueous HCl (5% v/v) (4 mL) and CH_3CN (4 mL) was mixed with a drop of saturated aqueous ammonium thiocyanate. Samples that showed a red color after 15 min were treated with Amberlite IRC748 until a colorless test was obtained.

Endomorphin-2 (2). From 308 mg (0.194 mmol) of aminoacyl resin prepared according to procedure A. Peptide chain was elongated until the *N*-terminal end following general procedures B and C. The *C*-terminal allyl group was then removed following general procedure D, and H-Phe-NH_2 (0.10 g, 0.61 mmol), DIC (91 μL, 0.59 mmol), oxyme (0.08, 0.58 mmol) in DMF (8 mL) were added. The mixture was reacted for 1 h at rt with occasional manual stirring (this procedure was repeated twice to ensure the complete coupling), and the *N*-terminal Fmoc group was removed according to general procedure C. Subsequent peptide cleavage from the resin (98% yield) and diazonium salt reduction (general procedure E) afforded a crude material (73.1% of purity by HPLC) that was treated with the mixture TFA/TIS/H_2O (95/2.5/2.5 v/v) for 3 h at rt. Volatiles were removed under vacuum, the product was precipitated with Et_2O, and the solid was centrifuged (10 min at 3500 rpm) and filtered. Endomorphin-2 was purified by semipreparative reversed-phase HPLC (5% → 15% B in 1 min and 15% → 23% in 5 min with a flow rate of 16 mL/min and 214 nm UV detection, t_R = 4.90 min), yielding 26.1 mg of **2** (global yield of 38%). ESI-HRMS calcd for $C_{32}H_{38}N_5O_5$ $[M + H]^+$, 572,2873; found 572,2861. HPLC analysis was performed with 220 nm UV detection on a C18 analytical column (4.6 × 100 mm) eluting with a gradient of 5% → 100% B over 11 min with a flow rate of 2 mL/min. t_R = 4.65 min.

Ac-AAPF-pNA (3). From 310 mg (0.195 mmol) of aminoacyl resin prepared according to procedure A. The general procedure D was followed to remove the allyl group and the resin was treated five times with *p*-nitroaniline (0.3 g, 2.17 mmol), PyAOP (0.3 g, 0.58 mmol), HOBt (0.08 g, 0.59 mmol), and DIEA (200 μL, 1.15 mmol) in DMF (8 mL) for 1 h at rt. Then, the mixture was washed with DMF (5 × 30 s), DCM (5 × 30 s), MeOH (5 × 30 s), and DCM (5 × 30 s). The peptide chain was further assembled (general procedure B), the *N*-terminal Fmoc group was removed (general procedure C), and finally, acetylation was performed (general procedure A). Peptide cleavage from the resin (96% yield) and diazonium salt reduction (general procedure E) afforded a peptide crude (86.4% of purity by HPLC) that was purified by semipreparative reversed-phase HPLC (5% → 35% B in 1 min and 35% → 53% in 5 min with a flow rate of 16 mL/min and 214 nm UV detection, t_R = 4.92 min), yielding 12.8 mg of **3** (global yield of 19%). ESI-HRMS calcd for $C_{28}H_{35}N_6O_7$ $[M + H]^+$, 567,2567; found, 567,2586. HPLC analysis was performed with 220 nm UV detection on a C18 analytical column (4.6 × 100 mm) eluting with a gradient of 5% → 100% B over 11 min with a flow rate of 2 mL/min. t_R = 6.35 min.

Biotynilated Ac-AAPF (4). From 301 mg (0.190 mmol) of aminoacyl resin prepared according to procedure A. The peptide chain was grown according to general procedures B and C when acetylation was carried out as described in general procedure A, and the *C*-terminal allyl group was removed following the general procedure D. Then, mono-Fmoc-ethylene diamine hydrochloride (0.30 g, 0.95 mmol), DIC (90 μL, 0.58 mmol), oxyme (0.08 g, 0.57 mmol), and DIEA (165 μL, 0.95 mmol) in DMF/DCM (1/1 v/v) (8 mL) were added. The mixture was left for 1 h with occasional manual stirring. The *N*-terminal Fmoc group was then removed using the general procedure C, and biotin (0.14 g, 0.57 mmol) was added together with PyAOP (0.3 g, 0.58 mmol), HOAt (0.08 g, 0.59 mmol), and DIEA (200 μL, 1.15 mmol) in DMF/DCM (1/1 v/v) (8 mL). The mixture was left for 1 h with occasional manual stirring, and the resin was washed with DMF (5 × 30 s), DCM (5 × 30 s), MeOH (5 × 30 s), and DCM (5 × 30 s). Peptide cleavage from the resin (97% yield) and diazonium salt reduction (general procedure E) allowed the desired product (93.4% of purity by HPLC) that was purified by semipreparative reversed-phase HPLC (5% → 20% B in 1 min and 20% → 33% B in 5 min with a flow rate of 16 mL/min over 7 min and 214 nm UV detection, t_R = 4.35 min) to be obtained, yielding 26.9 mg of **4** (global yield of 32%). ESI-HRMS: calcd for $C_{34}H_{51}N_8O_7S$ $[M + H]^+$ 715.3601, found, 715.3587. HPLC analysis was performed with 220 nm UV detection on a C18 analytical column (4.6 × 100 mm) eluting with a gradient of 5% → 100% B over 11 min with a flow rate of 2 mL/min. t_R = 4.45 min.

Schizandrochin B (5). From 270 mg (0.170 mmol) of aminoacyl resin prepared according to procedure A. After removal of the allyl group (general procedure D), H-Pro-OMe·HCl (0.07 g, 0.54 mmol), PyAOP (0.26 g, 0.50 mmol), HOAt (0.07 g, 0.51 mmol), and DIEA (265 μL, 1.52 mmol) in DMF (8 mL) were added. The mixture was left for 1 h at rt when the Fmoc group was removed following the general procedure C (four times). Then, the mixture was washed with DMF (5 × 30 s), DCM (5 × 30 s), MeOH (5 × 30 s), and DCM (5 × 30 s). Peptide cleavage from the resin (>99% yield) and diazonium salt reduction (general procedure E) afforded the title peptide (79% of purity by HPLC) that was purified by semipreparative reversed-phase HPLC (5% → 20% B in 1 min and 20% → 33% B in 5 min with a flow rate of 16 mL/min and 214 nm UV detection, t_R = 4.62 min), yielding 17.1 mg of **5** (global yield of 67%). ESI-HRMS calcd for $C_{14}H_{17}N_2O_2$ $[M + H]^+$ 245.1290, found 245.1287. HPLC analysis was performed with 220 nm UV detection on a C18 analytical column (4.6 × 100 mm) eluting with a gradient of 5% → 100% B over 11 min with a flow rate of 2 mL/min. t_R = 4.45 min.

Unguisin A (6). From 320 mg (0.202 mmol) of aminoacyl resin prepared according to procedure A. After removal of the Fmoc group (general procedure C), the peptide chain was assembled following the general procedure B except for removal of the Fmoc group after anchoring GABA and further amino acids, where a mixture of DBU (10% v/v) and piperidine (10% v/v) in DMF (1 × 1 min and 2 × 10 min) was used. Then the *C*-terminal allyl group and *N*-terminal Fmoc group were removed following the general procedure D and using DBU (3% v/v) in DMF, respectively. Cyclization was carried out with two treatments of PyAOP (0.32 g, 0.61 mmol), HOAt (0.08 g, 0.59 mmol), and DIEA (212 μL, 1.22 mmol) in DMF (8 mL) for 1 h at rt with occasional manual stirring. Then, the resin was washed with DMF (5 × 30 s), DCM (5 × 30 s), MeOH (5 × 30 s), and DCM (5 × 30 s). Peptide cleavage from the resin (76% yield) and diazonium salt reduction (general procedure E) gave the desired product (84.2% of purity by HPLC) that was purified by semipreparative reversed-phase HPLC (5% → 35% B in 1 min and 35% → 48% B in 5 min with a flow rate of 16 mL/min and 214 nm UV detection, t_R = 4.90 min), yielding 28.7 mg of **6** (global yield of 30%). ESI-HRMS calcd for $C_{40}H_{55}N_8O_7$ $[M + H]^+$, 759,4194; found, 759,4180. HPLC analysis was performed with 220 nm UV detection on a C18 analytical column (4.6 × 100 mm) eluting with a gradient of 5% → 100% B over 13 min with a flow rate of 2 mL/min. t_R = 6.23 min.

Protected Hemorphine-7 Derivatives (7 and 8). From 800 mg (0.504 mmol) of aminoacyl resin prepared according to procedure A. After removal of Fmoc group of phenylalanine (general procedure C), the peptide chain was assembled following the general procedure B obtaining the desired peptidyl resin fully protected (peptidyl resin A).

Protected Hemorphine-7 Derivative 7. Peptidyl resin A (250 mg, 0.158 mmol) was used in the synthesis. The *C*-terminal allyl group was removed following the general procedure D, and the resin was washed with DMF (5 × 30 s), DCM (5 × 30 s), MeOH (5 × 30 s), and DCM (5 × 30 s). Peptide cleavage from the resin (98% yield) and diazonium salt reduction (general procedure E) gave the desired product (93.4% of purity by HPLC) that was purified by semipreparative reversed-phase HPLC (5% → 95% B in 1 min, 95% → 100% B in 5 min and 100% B in 5 min with a flow rate of 16 mL/min and 214 nm UV detection, t_R = 6.60 min), yielding 106.4 mg of **7** (global yield of 34.5%). ESI-HRMS: calcd for $C_{109}H_{129}N_{12}O_{18}S$ $[M + H]^+$ 1925,9269, found 1925.9248. HPLC analysis was performed with 220 nm UV detection on a C18 analytical column (4.6 × 100 mm) eluting with a gradient of 5% → 100% B over 13 min with a flow rate of 2 mL/min. t_R = 10.57 min.

*Protected Hemorphine-7 Derivative **8**.* Peptidyl resin A (250 mg, 0.158 mmol) was used in the synthesis. After the peptidyl resin was washed with DMF (5 × 30 s), *N*-terminal Fmoc was removed by treatment with DBU (3% v/v) in DMF (1 × 1 min and 2 × 10 min) and then the resin washed with DMF (5 × 30 s), DCM (5 × 30 s), MeOH (5 × 30 s) ,and DCM (5 × 30 s). Peptide cleavage from the resin (98% yield) and diazonium salt reduction (general procedure E) gave the desired product (94% of purity by HPLC) that was purified by semipreparative reversed-phase HPLC (5% → 10% B in 1 min and 10% → 25% B in 5 min with a flow rate of 16 mL/min and 214 nm UV detection, t_R = 4.30 min), yielding 88.1 mg of **8** (global yield of 32%). ESI-HRMS: calcd for $C_{97}H_{123}N_{12}O_{16}S$ $[M + H]^+$ 1743.8901, found 1743.8860. HPLC analysis was performed with 220 nm UV detection on a C18 analytical column (4.6 × 100 mm) eluting with a gradient of 5% → 100% B over 13 min with a flow rate of 2 mL/min. t_R = 9.87 min.

Racemization Study. *Synthesis of Model Peptides Ac-L-Phe-L-Phe-NH$_2$ and Ac-D-Phe-L-Phe-NH$_2$ (**9** and **10**).* Fmoc-Rink-amide MBHA resin (0.73 mmol/g, 0.73 mmol) was introduced into a polypropylene syringe fitted with a porous polystyrene frit and was washed successively with DCM (10 × 30 s) and DMF (10 × 30 s). Then, the Fmoc group was removed following general procedure C, and Fmoc-L-Phe-OH (0.85 g, 2.19 mmol), DIC (0.34 mL, 2.20 mmol), and oxyme (0.28 g, 2.19 mmol) in DMF (15 mL) were added. The mixture was reacted for 1 h at room temperature with occasional manual stirring. After removal of the Fmoc group of phenylalanine (general procedure C), the peptidyl resin was divided in two batches (A and B). Then, Fmoc-L-Phe-OH (batch A) and Fmoc-D-Phe-OH (batch B) were coupled using DIC (3 equiv) and oxyme (3 equiv) in DMF. After 1 h at room temperature with occasional manual stirring, the Fmoc group was removed following the general procedure C and acetylation performed following general procedure A. Diastereoisomeric peptides were cleaved from the corresponding resins using the acidolytic mixture TFA/TIS:DCM (95:2.5:2.5) for 2h. The crude products were precipitated with hexanes and the remaining solids were centrifuged (5 min at 3500 rpm) and filtered, yielding white solids that were analyzed by HPLC: C18 analytical column (4.6 × 100 mm) eluting at 220 nm with a gradient of 20% → 50% B over 4.5 min with a flow rate of 2 mL/min; t_R = 1.87 min (Ac-L-Phe-L-Phe-NH$_2$, **9**) and t_R = 2.23 min (Ac-D-Phe-L-Phe-NH$_2$, **10**).

*Synthesis of Model Peptide Ac-L-Phe-L-Phe-NH$_2$ (**9**) Using a Triazene Linkage. Synthetic Route **1**.* From 142 mg (0.09 mmol) of aminoacyl resin prepared according to general procedure A. After removal of Fmoc group (general procedure C), acetylation was performed following general procedure A. Then, *C*-terminal allyl group was removed following the general procedure D, and the resin was washed with DMF (5 × 30 s), DCM (5 × 30 s), MeOH (5 × 30 s) and DCM (5 × 30 s). Then H-Phe-NH$_2$·HCl (0.05 g, 0.27 mmol), DIC (40 μL, 0.26 mmol), and oxyme (0.03 g, 0.27 mmol) in DMF (8 mL) were added. The mixture was reacted for 1 h at rt with occasional manual stirring (this procedure was repeated twice to ensure the complete coupling). Finally, peptide cleavage from the resin and diazonium salt reduction (general procedure E) afforded peptide crude that was analyzed by HPLC.

*Synthetic Route **2**.* From 153 mg (0.1 mmol) of aminoacyl resin prepared according to general procedure A. The *C*-terminal allyl group was removed following general procedure D, and H-Phe-NH$_2$·HCl (0.06 g,0.30 mmol), DIC (46 μL, 0.30 mmol), and oxyme (0.04 g, 0.32 mmol) in DMF (8 mL) were added. The mixture was reacted for 1 h at rt with occasional manual stirring (this procedure was repeated twice to ensure the complete coupling), and the *N*-terminal Fmoc group was removed according to general procedure C. Finally, acetylation was performed following the general procedure D. Peptide cleavage from the resin and diazonium salt reduction (general procedure E) afforded peptide crude that was analyzed by HPLC.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C NMR spectra of phenylalanine derivatives, HPLC-MS data of crude peptides, HPLC and HRMS of pure peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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