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2-Mercaptopyridine-1-oxide-based peptide coupling reagents

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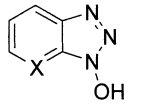
Abstract—The thiouronium salts *S*-(1-oxido-2-pyridinyl)-1,1,3,3-tetramethylthiouronium hexafluorophosphate (HOTT) and tetrafluoroborate (TOTT), and *S*-(1-oxido-2-pyridinyl)-1,3-dimethyl-1,3-trimethylenethiouronium hexafluorophosphate (HODT) and tetrafluoroborate (TODT), prepared from 2-mercaptopyridine-1-oxide and 1,1,3,3-tetramethylurea (TMU) or 1,3-dimethylpropyleneurea (DMPU), have been employed as reagents in solution and solid-phase peptide coupling chemistry. Furthermore, 2-mercaptopyridine-1-oxide has been employed as racemization-reducing additive combined with the new thiouronium salts and other frequently used peptide coupling reagents such as DCC or TBTU.

1. Introduction

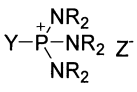
The formation of amide bonds is the main goal in the synthesis of a huge array of organic compounds of biological interest[1] such as peptides and peptoids, and other compounds containing the amide bond such as oligocarbamates, oligoamides, β-lactams, polyenamides, benzodiazepines, diketopiperazines, and hydantoins. The activation of carboxylic acids for the formation of the amide bond is an important process usually carried out using so-called peptide coupling reagents.[2] The coupling reactions can be carried out in solution or solid-phase by in situ activation of the carboxylic acid or by the prior preparation and isolation of an activated species. A commonly encountered problem in peptide synthesis involves the extensive racemization of the amino acid component, usually through oxazolone formation, and also undesired side reactions and low coupling rates especially under solid-phase conditions.[1c,2,3] Thus, a combination of high efficiency during the formation of the amide bond together with a low level of racemization of the coupled components represent the main challenge in the development of any new coupling reagent.

During the last few years, a plethora of coupling reagents has appeared in the literature,[2] considerably improving older coupling methods using, for example, carbodiimides.[4] These latter reagents, in the presence of several additives with an X–OH structure, such as 1-hydroxybenzotriazole (HOBt, **1a**),[5] 1-hydroxy-7-azabenzotriazole (HOAt, **1b**)[6] or hydroxylamine derivatives, such as free[3] or polymer-supported[7] *N*-hydroxysuccinimide (HOSu), are still very useful and widely used peptide coupling reagents.[4] Nowadays, the preferred peptide coupling reagents are principally phosphonium (**2**) or aminium (**3**) derivatives, where Y is a halogen or a XO group, which have become popular because of their higher efficiency and lower tendency towards racemization of the amino acid or peptide residues. Frequently employed peptide coupling reagents are exemplified by BOP[8] (**4a**) and TBTU[9] (**5a**), derived from HOBt and a phosphotriamide or a urea, and more recently by AOP[6b,10] (**4b**) and HATU[6a] (**5b**) derived from HOAt.

In general, both components of the coupling reagent, for instance the XOH and the urea, should be soluble in water to facilitate their separation from the final peptide formed especially when used in solution phase couplings. This particular characteristic prompted us to try 2-mercaptopyridine-1-oxide (**6**) as a XSH-derived species. Compound **6** is a cheap reagent, which is commercially available as an



1a; X = CH (HOBt)
1b; X = N (HOAt)



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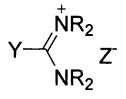

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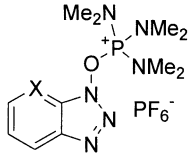
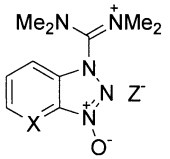
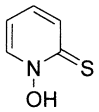


4a; X = CH (BOP)
4b; X = N (AOP)



5a; X = CH, Z=BF_4 (TBTU)
5b; X = N, Z=PF_6 (HATU)



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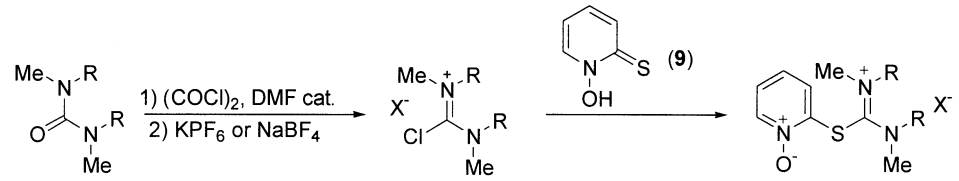


9; R = Me
10; R-R = $(CH_2)_3$

11; R = Me; **a**: X = PF_6; **b**: X = BF_4
12; R-R = $(CH_2)_3$; **a**: X = PF_6; **b**: X = BF_4

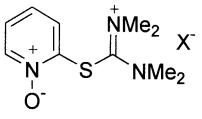
7a,**b**
8a,**b**

Scheme 1.

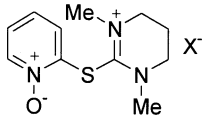
aqueous solution of its sodium salt, and has been used principally for the preparation of Barton thiohydroxamate esters, which are precursors of radicals generated by photolysis.[11]

We have recently employed 2-mercaptopyridine-1-oxide (**6**) and 1,1,3,3-tetramethylurea (TMU) as starting materials for the preparation of a new class of aminium peptide coupling reagents[12] with a thiouronium structure.[13] Thus, we prepared *S*-(1-oxido-2-pyridinyl)-1,1,3,3-tetramethylthiouronium hexafluorophosphate (HOTT, **7a**)[15] and the corresponding tetrafluoroborate (TOTT, **7b**), and used them for solution-phase peptide synthesis[12] achieving yields and levels of racemization comparable to other more expensive and commonly used coupling reagents. We have also employed these salts for the direct preparation of primary amides by the coupling of carboxylic acids with ammonium chloride.[16]

In addition, and taking into account the reported embryotoxicity of TMU,[17] we have also prepared the analogous thiouronium salts **8a** (HODT) and **8b** (TODT) from a nontoxic urea, the common organic solvent 1,3-dimethylpropyleneurea (DMPU).[18,19] The use of compounds **8** as peptide coupling reagents in solution[20] has been studied and their coupling characteristics have been compared to other reagents including **7**. The utility of the thiouronium salts **7** and **8** in solid-phase peptide synthesis (SPPS) employing hindered amino acids is also reported with particular emphasis being placed on their efficiency and level of racemization produced during the coupling reactions. We have also studied the use of compound **6** as an additive in combination with other peptide coupling reagents in order to check its effectiveness as a racemization-reducing agent.



7a; X = PF_6 (HOTT)
7b; X = BF_4 (TOTT)



8a; X = PF_6 (HODT)
8b; X = BF_4 (TODT)

2. Results and discussion

The uronium salts **7**[12] and **8**[18–20] can be prepared by the one-pot phosgene-free methodology outlined in Scheme 1. The thiouronium salts **8** were used as peptide coupling reagents for the liquid-phase preparation of a series of di- and tripeptides and the results compared with those obtained with

Table 1. Prepared peptides in solution-phase using **8** as coupling reagents

Entry	Reagent	*t* (h)	Peptide (no.)	Yield (%)[a]	Mp (°C)	$[\alpha]_D^{25}$
1	**8a**	4	Cbz-Val-Val-OMe (**13a**)	82	110–111	−22.6[b]
2	**8b**	4		83		−22.3[b]
3	**8a**	4	Cbz-Val-Aib-OMe (**13b**)	60	88–89	−20.0[c]
4	**8b**	4		76		−20.0[c]
5	**8b**	4	Boc-Val-Aib-OMe (**13c**)	66	148–149	−16.0
6	**8a**	4	Boc-Aib-Val-OMe (**13d**)	66	110–111	
7	**8b**	4		70		
8	**8b**	24	Cbz-Aib-Aib-OMe (**13e**)	74 (68)	109–110	
9	**8a**	24	Boc-Aib-Aib-OMe (**13f**)	66	86–87	
10	**8b**	24		83		
11	**8b**	24	Fmoc-Aib-Aib-OMe (**13g**)	47[d] (60[d])	127–128	
12	**8a**	4	Bz-Leu-Gly-OEt (**13h**)	64	151–152	−20.5[e]
13	**8b**	4		70		−21.0[e]
14	**8a**	4	Cbz-Gly-*Phe-Val*-OMe[f] (**13i**)	63[g]		
15	**8b**	4		84[h]		
16	**8a**	4	Cbz-MeVal-Val-OMe (**13j**)	81[d]		−71.5[i]
17	**8b**	4		75[d]		−73.2[i]

[a] Isolated pure compounds after aqueous work-up. Based in the starting amino acid. In parentheses, yields using **7b** as coupling reagent.
[b] Lit.[25a] $[\alpha]_D^{20}=-28$ (*c* 1, EtOH).
[c] Lit.[25b] $[\alpha]_D=-24$ (*c* 1, EtOH).
[d] After column chromatography (silica gel, hexane/AcOEt).
[e] Lit.[23] $[\alpha]_D^{20}=-34$ (*c* 3.1, EtOH).
[f] Underlined the new formed bond.
[g] Diastereomeric ratio (^{1}H NMR): 10:0.8.
[h] Diastereomeric ratio (^{1}H NMR): 10:1.
[i] Lit.[22b] $[\alpha]_D^{20}=-90$ (*c* 1, EtOH).

Table 2. Use of **6** as additive in Anteunis' test (synthesis of **13i**)

Entry	Reagent+**6** (1 equiv.)	Yield (%)[a]	D,L (%)[b]
1	DCC+**6**	81[c]	6.5
2	DCC		36[d]
3	TBTU (**5a**)+**6**	90	1.4
4	TBTU (**5a**)		1.4[d]
5	HOTT (**7a**)+**6**	87	3.8
6	HOTT (**7a**)	68	9.0
7	TOTT (**7b**)+**6**	83	2.4
8	TOTT (**7b**)	71	9.0
9	HODT (**8a**)+**6**	72	3.8
10	HODT (**8a**)	63	7.4
11	TODT (**8b**)+**6**	85	2.9
12	TODT (**8b**)	84	9.0

[a] Isolated pure compound after aqueous work-up. Based on the starting amino acids.
[b] Measured by ^{1}H NMR.
[c] The reactions was performed in CH_2Cl_2.
[d] Measured by HPLC (Ref. 9).

7. In all cases we checked the efficiency of these reagents performing rather difficult couplings using hindered amino acids such as Val or, specially, α,α-dialkyl amino acids such as α-aminoisobutyric acid (Aib) or *N*-methylated amino acids. The Aib–Aib coupling is described as being particularly difficult for both solution and solid-phase strategies,[21] whereas *N*-methylated amino acids usually prove resistant in coupling reactions.[22a]

Thus, different Boc-, Cbz-, Fmoc- and Bz-*N*-protected amino acids or dipeptides were reacted with ethyl or methyl ester amino acid hydrochlorides in the presence of the uronium salts **8** and triethylamine as base using DMF as solvent. After work-up, the corresponding pure (^{1}H NMR, 300 MHz) crude peptides **13** were isolated with the yields shown in Table 1. The reaction time was always 4 h, except when the difficult Aib–Aib coupling was performed, which required 24 h for completion. A certain tendency was observed for the hexafluorophosphate **8b** to afford higher yields than the corresponding tetrafluoroborate **8a**, a feature already observed with reagents **7**.[12] In general, the DMPU derivatives **8** afforded similar or even higher yields than the corresponding TMU derivatives **7**.[12] Moreover, *N*-Boc-protected amino acids are particularly prone to *N*-carboxyanhydride formation,[22b] and it is remarkable that the yield in the preparation of the 'difficult' dipeptide Boc-Aib-Aib-OMe was 83% when the DMPU-derivative **8b** was employed (Table 1, entry 10), but only 70% when TBTU (**5a**) was used for comparison. Furthermore, a *N*-methylated amino acid, such as Cbz-MeVal-OH afforded good isolated yields of the corresponding peptide **13j** when coupled with Val-OMe using **8** (Table 1, entries 16 and 17) values comparable to those obtained using **7** (80% for **7a** and 82% for **7b**).[12]

The extent of racemization under solution-phase conditions using these reagents was examined by performing Young's test[23] (the coupling of Bz-Leu-OH and Gly-OEt·HCl, Table 1, entries 12 and 13) and Anteunis' test[24] (the coupling of Cbz-Gly-Phe-OH and Val-OMe·HCl, Table 1, entries 14 and 15). A higher level of racemization was observed in Young's test using DMPU-derived reagents **8** when compared to the corresponding TMU derivatives **7**.[12] Thus, for reagents **8**, the level of racemization was ca. 19%, a value close to that obtained when using HATU (**5b**),[12] whereas the corresponding value was only 3.7% using reagent **7b**.[12] On the other hand, when Anteunis' test was performed, the DMPU-derived salt **8a** gave the lowest level of epimerization [diastereomeric ratio (^{1}H NMR)=10:0.8] in the corresponding tripeptide **13i** (Table 1, entry 14), reagents **7** giving slightly higher diastereomer ratios (10:1, NMR).[12]

The reactivity of peptide coupling reagents can be enhanced in some cases by the addition of various additives which at the same time also serve to reduce the extent of racemization.[1] For instance, a serious drawback to the use of carbodiimides as peptide coupling reagents is the high level of racemization usually observed in the coupled product. However, in the presence of additives with a XOH structure, usually a hydroxylamine derivative, yields are increased and racemization levels are lowered.[4,7] Moreover, it has been shown that racemization is decreased during the coupling of peptide fragments in solution when HOBt (**1a**) or HOAt (**1b**) or are added to aminium-derived coupling reagents.[26] These antecedents prompted us to explore the use of 2-mercaptopyridine-1-oxide (**6**) as an additive when combined with other reagents commonly used for peptide couplings reagents, and also with the thiouronium salts **7** and **8**. Thus, the Anteunis' tripeptide **13i** was obtained by using a mixture of **6** (1 equiv.) and a carbodiimide such as *N*,*N*-dicyclohexylcarbodiimide (DCC), affording a product with a dramatically lowered racemization level when compared to the values reported in the absence of any additive[9] (compare Table 2, entries 1 and 2), although not as much as the reported lowering when HOBt was used, which gave no epimerization at all.[24] However, when **6** was employed as additive in combination with TBTU (**5a**), no lowering of the racemization degree was observed when compared to the value measured without additive[9]

Table 3. Efficiency of the reagents **7** and **8** in SPPS

Entry	Reagent	Peptides	
		H-Tyr-Aib-Aib-Phe-Leu-NH_2, **14** (%)[a]	H-Tyr-Aib-Phe-Leu-NH_2, **15** (%)[a]
1	TBTU (**5a**)	29	71
2	HOTT (**7a**)	24	76
3	TOTT (**7b**)	19	81
4	HODT (**8a**)	10	90
5	TODT (**8b**)	14	86

[a] Measured by integration of the signals in the HPLC at 220 nm (see Section 3).

Table 4. Racemization studies in SPPS using **7** and **8** with Ser and His models

Entry	Reagent	Peptides	
		Gly-(L)-Ser-Phe-NH_2, **16** (%)[a]	Gly-(L)-His-Phe-NH_2, **17** (%)[a]
1	TBTU (**5a**)	>99	97.1
2	HOTT (**7a**)	>99	98.4
3	TOTT (**7b**)	>99	98.6
4	HODT (**8a**)	>99	98.1
5	TODT (**8b**)	>99	98.0

[a] Measured by integration of the signals in the HPLC at 220 nm (see Section 3).

(compare Table 2, entries 3 and 4). A significant effect was observed when an extra equivalent of **6** was used with reagents **7** or **8**, affording in all cases a decrease in racemized product and an increase in the final yield of the tripeptide (Table 2, entries 5–12).

In order to check the efficiency of **7** and **8** in SPPS, we performed the manual stepwise solid-phase assembly of a typical model pentapeptide analog of Leu-enkephalinamide, H-Tyr-Aib-Aib-Phe-Leu-NH_2 (**14**),[6b,27a] which contains Aib-OH residues at positions 2 and 3 instead of Gly-OH, following a Fmoc/*t*Bu protection scheme. A poly(ethyleneglycol)–polystyrene (PEG–PS)-resin bearing a peptide amide linker derived from *p*-{α-[1-(9*H*-fluoren-9-yl)methoxyformamido]-2,4-dimethoxybenzyl}phenoxyacetic acid (Rink) was used as a solid support, in DMF as solvent and using diisopropylethylamine (DIEA) as base (see 3). TBTU (**5a**) (five-fold excess, single coupling) was employed as coupling reagent for normal amino acids, whereas **7** or **8** (five-fold excess, single coupling) were used for the coupling of the difficult Aib-Aib sequence. Table 3 shows the amount of pentapeptide (**14**) and des-Aib tetrapeptide (**15**) obtained after cleavage, together with the result for Aib–Aib coupling using TBTU (**5a**) for comparison (Table 3, entry 1). In all cases the efficiency in the Aib–Aib coupling was lower than with TBTU, although the result was not very different when using the TMU-derived tetrafluoroborate **7a** (Table 3, entry 2). The lowest efficiency was observed with the DMPU-derived thiouronium **8a** (Table 3, entry 4).

The extent of racemization induced by these reagents in SPPS was studied performing the manual stepwise solid-phase assembly of two model tripeptides containing Ser (H-Gly-Ser-Phe-NH_2, **16**)[27b] and His (H-Gly-His-Phe-NH_2, **17**) both amino acids especially prone to racemization.[27c,d] The synthesis was performed following the same methodology as above, using TBTU (**5a**) except when coupling the Ser or His residues to Phe where **7** or **8** were used. In both cases, an all-TBTU (**5a**) synthesis was including for comparison (Table 4, entry 1). The extent of racemization was obtained by measuring the ratio between diastereomeric peptides containing L- and D-Ser, and L- and D-His (HPLC), the yield of the L-containing peptides being presented (Table 4). The extent of racemization in the Ser-containing model tripeptide (**16**) was very low in all cases and similar to that observed using TBTU (**5a**) (Table 4). However, for the more sensitive His-containing tripeptide (**17**), the racemization when using reagents **7** and **8** was lower in all cases when compared to TBTU (**5a**) (Table 4), especially when the thiopyridone derivatives **7** were used (Table 4, entries 2 and 3).

In conclusion, 2-mercaptopyridine-1-oxide-derived thiouronium salts from TMU [HOTT (**7a**) and TOTT (**7b**)] or DMPU [HODT (**8a**) and TODT (**8b**)] can be prepared following an easily scalable one pot procedure from economical reagents and can be employed as efficient peptide coupling reagents. The coupling of hindered amino acids in solution using these reagents affords generally higher yields for the DMPU derivatives **8** when compared to the TMU derivatives **7**. However, the results obtained in SPPS reveal a higher efficiency for the TMU derivatives **7**, together with lower racemization levels. This could be explained by the fact that, in general, DMPU derivatives are less stable than the corresponding TMU derivatives[3] and in solid-phase the reaction usually has lower kinetics. The addition of an extra amount of 2-mercaptopyridine-1-oxide to the thiouronium salts **7** and **8** improves the final yields and lowers lowering the extent of racemization. The results obtained show that the reagents **7** and **8** are comparable to other peptide coupling reagents presently available.

3. Experimental

3.1. General

All reactions carried out during the preparation of the thiouronium salts were moisture-protected using a drying tube with calcium chloride. 2-Mercaptopyridine-1-oxide (**6**) was obtained according to the literature procedure.[28] Reagents and solvents from commercial suppliers were used as provided. PEG–PS resin (0.19 mmol g^{-1}) and the Fmoc-Rink handle were obtained from the BioSearch division of PerSeptive Biosystems (Framingham, MA). Melting points are uncorrected. IR spectra were recorded with a Nicolet 510 P-FT and only the structurally important peaks are indicated. NMR spectra were measured with a Bruker AC-300 at 300 MHz for ^{1}H and 75 MHz for ^{13}C using TMS as internal standard. Optical rotations were measured with a Jasco DIP-1000 polarimeter. MALDI-TOF spectra were recorded with a Perspective Biosystems Voyager DE™-RP spectrometer using a 2,5-dihydroxybenzoic acid matrix. Analytical TLC was performed with Schleicher and Schuell F1400/LS silica gel plates and the spots were visualized with UV light at 254 nm or by charring with 0.3% ninhydrin. Flash chromatography was carried out with Merck silica gel 60 (0.040–0.063 nm).

Reverse-phase HPLC analyses were carried out on Nucleosil C18 columns (25×0.5 cm, 10 μm) using linear gradients of water/0.045% TFA (solvent A) and MeCN/0.036% TFA (solvent B) (flow: 1 mL min^{-1}, detection wavelength: 220 nm). Leu-enkephalinamide derivative **14** was analysed using a linear gradient from 25 to 30% of B in 30 min followed by 100% of B for 5 min. The Ser and His racemization models **16** and **17** were analysed using a linear gradient from 0 to 20% of B in 20 min, followed by a gradient from 20 to 100% of B in 20 min and 100% of B for 5 min. The HPLC signals were measured at 220 nm.

3.2. Synthesis of thiouronium salts 7 and 8

S-(1-Oxido-2-pyridinyl)-1,1,3,3-tetramethylthiouronium hexafluorophosphate (HOTT, **7a**) and the corresponding tetrafluoroborate (TOTT, **7b**) were prepared as reported.[12] *S*-(1-Oxido-2-pyridinyl)-1,3-dimethylpropylenethiouronium hexafluorophosphate (HODT, **8a**) and the corresponding tetrafluoroborate (TODT, **8b**) were prepared as follows: to a solution of 1,3-dimethylpropyleneurea (DMPU) (4.8 mL, 40 mmol) and DMF (0.3 mL) in CH_2Cl_2 (40 mL) was added dropwise oxalyl chloride (4.2 mL, 48 mmol) at room temperature. The solution was stirred for 1 h at room temperature and then refluxed for 24 h. The solvent was evaporated (15 Torr) and the resulting solid was stirred with portions of CH_2Cl_2 (2×10 mL) followed by evaporation of the organics (15 Torr) after each treatment. The crude chlorouronium salt obtained was dissolved in MeCN (40 mL) and KPF_6 (8.8 g, 48 mmol) or $NaBF_4$ (5.27 g, 48 mmol) were added. The mixture was stirred at room temperature for 24 h and to the resulting suspension was added *N*-hydroxy-2-pyridinethione (**6**) (5.1 g, 40 mmol). Triethylamine (6.7 mL, 48 mmol) was added dropwise keeping the temperature below 25°C and the resulting suspension was stirred at room temperature for 4 h and at 50°C for 1 h. The suspension was filtered through a plug of celite, the solvents were evaporated (15 Torr) and the thiouronium salt **8a** was obtained after crystallization with MeOH/isopropanol (12.4 g, 81%). Compound **8b** was isolated after stirring in EtOH at 40°C for 1 h, cooling at 4°C and filtration (7.8 g, 60%).

3.2.1. *S*-(1-Oxido-2-pyridinyl)-1,3-dimethylpropyleneuronium hexafluorophosphate (HODT, 8a). Mp 111–112°C. IR (KBr) ν 1629 (C═N), 844 (PF_6^-) cm^{-1}. ^1H NMR (CD_3CN) δ 2.21–2.23 (m, 2H, C$H_2$$CH_2N$), 3.37 (s, 6H, CH_3), 3.65 (t, *J*=6.1 Hz, 4H, CH_2N), 7.35–7.47 (m, 3H, ArH), 8.30 (d,1H, *J*=5.5 Hz, ArH). ^{13}C NMR (CD_3CN) δ 20.4 (1C, CH_2), 44.7 (2C, CH_3), 51.6 (2C, CH_2), 125.4, 126.2, 128.8, 140.2, 144.4 (5C, ArC), 160.4 (1C, C═N). Anal. Calcd for $C_{11}H_{16}N_3OSPF_6$: C, 34.47; H, 4.21; N, 10.96. Found: C, 34.28; H, 4.29; N, 11.02.

3.2.2. *S*-(1-Oxido-2-pyridinyl)-1,3-dimethylpropyleneuronium tetrafluoroborate (TODT, 8b). Mp 121–122°C. IR (KBr) ν 1620 (C═N), 1025 (BF_4^-) cm^{-1}. ^1H NMR (CD_3CN) δ 2.20–2.23 (m, 2H, C$H_2$$CH_2N$), 3.37 (s, 6H, CH_3), 3.65 (t, *J*=6.1 Hz, 4H, CH_2N), 7.34–7.47 (m, 3H, ArH), 8.30 (d, *J*=5.5 Hz, 1H, ArH). ^{13}C NMR (CD_3CN) δ 20.4 (1C, CH_2), 44.8 (2C, CH_3), 51.7 (2C, CH_2), 125.4, 126.2, 128.8, 140.2, 144.4 (5C, ArC), 160.4 (1C, CN). Anal. Calcd for $C_{11}H_{16}N_3OSBF_4$: C, 40.60; H, 4.96; N, 12.92. Found: C 40.52; H, 4.32; N, 12.60.

3.3. General procedure for the peptide coupling reactions in solution-phase

A solution of the *N*-protected amino acid (1 mmol), the amino acid ester hydrochloride (1 mmol), triethylamine (0.28 mL, 2 mmol) and the coupling reagent **8** (1 mmol) in DMF (5 mL) was stirred at room temperature until disappearance of the free esterified amino acid (TLC, ninhydrin). Saturated NaCl (25 mL) was added, and the mixture was extracted with AcOEt (3×25 mL). The organic layers were washed with 2N HCl (2×10 mL), saturated $NaHCO_3$ (2×10 mL) and water (6×10 mL). The organics were dried (Na_2SO_4), filtered and evaporated (15 Torr) affording the corresponding peptides **13** (see Table 1).

3.3.1. Cbz-Val-Val-OMe (13a).[25a] ^1H NMR ($CDCl_3$) δ 0.87–0.98 (m, 12H, 2(CH_3)$_2$CH), 2.04–2.19 (m, 2H, 2CH(CH_3)$_2$), 3.73 (s, 3H, CH_3O), 4–4.10 (m, 1H, CHCONH), 4.55 (dd, *J*=8.5, 4.9 Hz, 1H, C$H$$CO_2Me$), 5.12 (s, 2H, CH_2), 5.41 (d, *J*=8.5 Hz, 1H, NH), 6.44 (d, *J*=8 Hz, 1H, NH), 7.34 (m, 5H, ArH).

3.3.2. Cbz-Val-Aib-OMe (13b).[25b] ^1H NMR ($CDCl_3$) δ 0.94 (2d, *J*=6.7 Hz, 6H, (CH_3)$_2$CH), 1.51 (s ancho, 6H, (CH_3)$_2$C), 2.10 (m, 1H, CH(CH_3)$_2$), 3.70 (s, 3H, CH_3O), 3.96 (dd, *J*=14, 7 Hz, 1H, CHCO); 5.10 (s, 2H, CH_2), 5.56, 6.75 (2 broad s, 2H, 2NH), 7.29–7.35 (m, 5H, ArH).

3.3.3. Boc-Val-Aib-OMe (13c).[29] ^1H NMR ($CDCl_3$) δ 0.85 (2d, *J*=7 Hz, 6H, (CH_3)$_2$CH), 1.37–1.45 (3s, 15H, (CH_3)$_3$C, (CH_3)$_2$C), 2.10 (m, 1H, CH(CH_3)$_2$), 3.65 (s, 3H, CH_3O), 4.45 (dd, *J*=8.5, 4.9 Hz, 1H, CHCO), 4.97, 6.96 (2 broad s, 2H, 2NH).

3.3.4. Boc-Aib-Val-OMe (13d).[29] ^1H NMR ($CDCl_3$) δ 0.90 (2d, *J*=7 Hz, 6H, (CH_3)$_2$CH), 1.44–1.52 (3s, 15H, (CH_3)$_3$C, (CH_3)$_2$C), 2.16 (m, 1H, CH(CH_3)$_2$), 3.72 (s, 3H, CH_3O), 4.52 (dd, *J*=8.5, 4.9 Hz, 1H, CHCO), 4.93, 7.02 (2 broad s, 2H, 2NH).

3.3.5. Cbz-Aib-Aib-OMe (13e).[25b] ^1H NMR ($CDCl_3$) δ 1.50, 1.51 (2s, 12H, 2(CH_3)$_2$C), 3.71 (s, 3H, CH_3O), 5.10 (s, 2H, CH_2), 5.34, 6.90 (2 broad s, 2H, 2NH), 7.30–7.40 (m, 5H, ArH).

3.3.6. Boc-Aib-Aib-OMe (13f).[25b] ^1H NMR ($CDCl_3$) δ 1.45 (s, 9H, (CH_3)$_3$C), 1.47, 1.54 (2s, 12H, 2(CH_3)$_2$C), 3.72 (s, 3H, CH_3O), 5.10, 7.10 (2 broad s, 2H, 2NH).

3.3.7. Fmoc-Aib-Aib-OMe (13g).[29] ^1H NMR ($CDCl_3$) δ 1.50, 1.52 (2s, 12H, 2(CH_3)$_2$C), 3.70 (s, 3H, CH_3O), 4.19 (t, *J*=6.7 Hz, 1H, CH), 4.39 (d, *J*=6.7 Hz, 2H, CH_2CH), 5.55, 6.90 (2 broad s, 2H, 2NH), 7.26–7.75 (m, 8H, ArH).

3.3.8. Bz-Leu-Gly-OEt (13h).[23] ^1H NMR ($CDCl_3$) δ 0.98 (d, *J*=6.1 Hz, 6H, (CH_3)$_2$CH), 1.26 (t, *J*=7.3 Hz, 3H, C$H_3$$CH_2$), 1.65–1.84 (m, 3H, C$H_2$CH, ($CH_3$)C$H$)), 4–4.10 (m, 2H, C$H_2$NH), 4.16–4.24 (q, 2H, *J*=7.3 Hz, C$H_2$$CH_3$), 4.74 (m, 1H, C$H$NH), 6.60, 6.71 (2 broad s, 2NH), 7.41–7.80 (m, 5H, ArH).

3.3.9. Cbz-Gly-Phe-Val-OMe (13i).[24] L-Phe-containing epimer: ^{1}H NMR ($CDCl_3$) δ 0.75 (2d, J=7 Hz, 6H, $(CH_3)_2CH$), 1.96 (m, 1H, $CH(CH_3)_2$), 2.94 (m, 2H, CH_2Ph), 3.57 (s, 3H, CH_3O), 3.77 (d, J=5.5 Hz, 2H, $HNCH_2CO$), 4.34 (dd, J=8, 5.5 Hz, 1H, HNC*H*-*i*Pr), 4.75 (m, 1H, C*H*-Bn), 5.01 (s, 2H, CH_2OCONH), 5.75 (t, J=5.5 Hz, 1H, $NHCH_2$), 6.86 (d, J=8 Hz, 1H, N*H*CH-*i*Pr), 7.04–7.26 (m, 10H, 9ArH, NH).

3.3.10. Cbz-MeVal-Val-OMe (13j).[22b] ^{1}H NMR ($CDCl_3$) δ 0.76–0.96 (m, 12H, $2(CH_3)_2CH$), 2.10, 2.30 (2m, 2H, $2CH(CH_3)_2$), 2.90 (s, 3H, CH_3N), 3.71 (s, 3H, CH_3O), 4.10 (m, 1H, C*H*NMe), 4.48 (dd, J=8.8, 5.2 Hz, C*H*NHCO), 5.17 (AB, J=11.6, 12.8 Hz, 2H, CH_2), 6.55 (d, J=8.8 Hz, 1H, NH), 7.36 (m, 5H, ArH).

3.4. General procedure for the peptide coupling reactions using 6 as additive

A solution of the *N*-protected amino acid (1 mmol), the amino acid ester hydrochloride (1 mmol), triethylamine (0.28 mL, 2 mmol), the coupling reagent (1 mmol) and 2-mercaptopyridine-1-oxide (**6**) (127 mg, 1 mmol) in DMF (5 mL) was stirred at room temperature for 4 h. Saturated NaCl (25 mL) was added, and the mixture was extracted with AcOEt (3×25 mL). The organic layers were washed with 2N HCl (2×10 mL), saturated $NaHCO_3$ (2×10 mL) and water (6×10 mL). The organics were dried (Na_2SO_4), filtered and evaporated (15 Torr) affording the peptide **13i** (see Table 2).

3.5. SPPS of Leu-enkephalinamide derivative 14

To a neutralized PEG–PS resin (1 g, 0.19 mmol g^{-1}) was added the preformed Fmoc-Rink handle (0.2 g, 0.38 mmol), TBTU (0.31 g, 5 equiv.), HOAt (0.13 g, 5 equiv.), and DIEA (0.323 mL, 10 equiv.) dissolved in DMF (2 mL). After overnight coupling, the resulting Fmoc-Rink–PEG–PS resin was washed with DMF (5×10 mL) and the Fmoc group was removed with 20% piperidine in DMF (5 mL, 1 min+5 mL, 15 min), followed by washes with DMF (5×10 mL), CH_2Cl_2 (5×10 mL) and MeOH (3×10 mL) and drying in vacuo at 25°C. The obtained resin (1 g) was pre-conditioned with DMF (5×10 mL) and a solution of Fmoc-Leu-OH (0.34 g, 5 equiv.), TBTU (0.31 g, 5 equiv.) and DIEA (0.323 mL, 10 equiv.) in DMF (1 mL) was added stirring the mixture for 2 h and washing the resin with DMF (5×10 mL) and CH_2Cl_2 (3×10 mL). The Fmoc group was removed with 20% piperidine in DMF (5 mL, 1 min+5 mL, 15 min), followed by washes with DMF (5×5 mL) and CH_2Cl_2 (3×5 mL). Following the same procedure, the Phe and first Aib residues were introduced using Fmoc-Phe-OH and Fmoc-Aib-OH. Qualitative ninhydrin test was used to determine completion of all couplings. The obtained Aib-Phe-Leu-Rink–PEG–PS resin was washed with MeOH (3×5 mL) and dried in vacuo at 25°C. To a portion of 50 mg of this resin was added a mixture of Fmoc-Aib-OH (15 mg, 5 equiv.), DIEA (8 μL, 10 equiv.) and the coupling reagents **7**, **8**, or TBTU (5 equiv.) in DMF (0.5 mL). The mixture was stirred at 25°C for 2 h and the resin was washed with DMF (5×2 mL) and CH_2Cl_2 (3×2 mL). Deprotection of the Fmoc was performed with 20% piperidine in DMF (2 mL, 1 min+2 mL, 15 min), followed by washes with DMF (5×2 mL) and CH_2Cl_2 (3×2 mL). The formed resin was washed with DMF (5×2 mL) and treated with a mixture of Fmoc-Tyr(*t*Bu)-OH (22 mg, 5 equiv.), TBTU (15 mg, 5 equiv.) and DIEA (8 μL, 10 equiv.) in DMF (0.5 mL). After stirring at 25°C for 2 h, the Fmoc group was deprotected with 20% piperidine in DMF (2 mL, 1 min+2 mL, 15 min), followed by washes with DMF (5×2 mL) and CH_2Cl_2 (3×2 mL). Final cleavage was achieved after swelling the obtained Tyr-Aib-Aib-Phe-Leu-Rink-PEG–PS resin with CH_2Cl_2 (3×2 mL) and treatment with TFA/triethylsilane (TES)/H_2O (95:3:2, 0.5 mL) with stirring for 2 h at 25°C. The suspension was filtered and the solvent was removed under a stream of nitrogen. The resulting residue was triturated with *terc*-butyl methyl ether and cooled in a freezer overnight. The mixture was centrifuged (4000 rpm) for 10 min and the solvent was decanted. The resulting solid was dried under a stream of nitrogen and liophilized giving a mixture of peptides **22** and **23** which was analyzed by HPLC (see Table 3).

3.5.1. Tyr-Aib-Aib-Phe-Leu-NH_2 (14). HPLC: t_R=16 min. MALDI-TOF MS calcd for $C_{32}H_{46}N_6O_6$: 610.7. Found: 611.3 (M^++H).

3.5.2. Tyr-Aib-Phe-Leu-NH_2 (15). MALDI-TOF MS calcd for $C_{28}H_{39}N_5O_5$: 525.6. Found: 526.5 (M^++H). HPLC: t_R=17 min.

3.6. SPPS of tripeptides 16 and 17

To a Rink PEG–PS resin prepared as in Section 3.5. (1 g), was added a solution of Fmoc-Phe-OH (0.37 g, 5 equiv.), TBTU (0.31 g, 5 equiv.) and DIEA (0.323 mL, 10 equiv.) in DMF (1 mL). The mixture was stirred for 2 h and the resin was washed with DMF (5×10 mL) and CH_2Cl_2 (3×10 mL). A qualitative ninhydrin test was used to determine completion of all couplings. The Fmoc group was removed with 20% piperidine in DMF (10 mL, 1 min+10 mL, 15 min), followed by washes with DMF (5×10 mL) and CH_2Cl_2 (3×10 mL). Portions of this Phe-Rink–PEG–PS resin (50 mg) were treated with a solution of DIEA (8 μL, 10 equiv.), the coupling reagent **7**, **8** or TBTU (5 equiv.) and Fmoc-Ser(*t*Bu)-OH (18 mg, 5 equiv., for the synthesis of **16**) or Fmoc-His(Trt)-OH (29 mg, 5 equiv., for the synthesis of **17**) in DMF (0.5 mL), and the mixture was stirred at 25°C for 2 h. The resin was washed with DMF (5×10 mL) and CH_2Cl_2 (3×10 mL) and the Fmoc was removed with 20% piperidine in DMF (2 mL, 1 min+2 mL, 15 min), followed by washes with DMF (5×10 mL) and CH_2Cl_2 (3×10 mL). The resulting resin was treated with a mixture of Fmoc-Gly-OH (14 mg, 5 equiv.), TBTU (15 mg, 5 equiv.) and DIEA ((8 μL, 10 equiv.) in DMF (0.5 mL). After stirring at 25°C for 2 h, the Fmoc group was deprotected with 20% piperidine in DMF (2 mL, 1 min+2 mL, 15 min), followed by washes with DMF (5×2 mL) and CH_2Cl_2 (3×2 mL). Final cleavage and treatment as in Section 3.5 afforded peptides **16** or **17** which were analyzed by HPLC.

3.6.1. Gly-Ser-Phe-NH_2 (16). HPLC: t_R=14.7 min (with L-Ser). MALDI-TOF MS calcd for $C_{14}H_{20}N_4O_4$: 308.4. Found: 308.6 (M^+).

3.6.2. Gly-His-Phe-NH_2 (17). HPLC t_R=15.5 min (with L-His), 17 min (with D-His). MALDI-TOF MS calcd for $C_{17}H_{22}N_6O_3$: 358.3. Found: 358.5 (M^+).

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