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A Novel Family of Onium Salts Based Upon Isonitroso Meldrum's Acid Proves Useful as Peptide Coupling Reagents

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A new family of uronium salts (HTMU, HMMU, and HDmPyMU) based on isonitroso Meldrum's acid (HONM) are reported as stand-alone coupling reagents. Amide bond formation with the use of these reagents occurred more quickly than that with other uronium salts as a result of the presence of a neighboring group effect with a cyclic structure. Thus, these novel onium salts were often more effective in the acylation of poor nucleophiles and in the control of optical purity than related Oxyma- and benzotriazole-based reagents. Among the HONM derivatives, HMMU showed the best performance in reducing racemization and assembling demanding sequences such as the Aib-ACP decapeptide or analogues of Leu-enkephalin pentapeptide. Furthermore, the scope and limitations of the use of HONM as an additive in combination with carbodiimides is discussed.

Introduction

The reaction of a carboxylic acid with an amine to render an amide requires activation of the carboxylic acid. This is very often carried out through an active ester, which can be previously prepared, isolated, purified, and characterized, or prepared in situ by the use of carbodiimides or onium salts. In all cases, the cornerstone of the process is the leaving group. The most used groups are those derived from phenols and *N*-hydroxy derivatives.[1] These include 1-hydroxybenzotriazole (HOBt, **1a**),[2] 7-aza-1-hydroxybenzotriazole (HOAt, **1b**),[3] 6-chloro-1-hydroxybenzotriazole (Cl-HOBt, **1c**),[4] and recently ethyl 2-cyano-2-(hydroxyimino)-acetate (Oxyma, **2**)[5] (Figure 1). Among the current reagents of choice for peptide[6] bond formation are aminium/uronium derivatives (also known as Knorr reagents).[7] These compounds have become popular because of their high efficiency and low tendency to induce racemization of the activated amino acid or peptide residue.

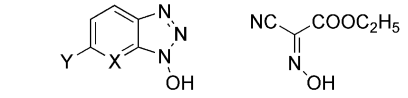

Figure 1. Structures of the carbodiimide additives HOBt, HOAt, Cl-HOBt, and Oxyma.

In general, amide bond formation mediated by uronium salts and other onium salts involves two steps: activation, in which the coupling reagent reacts with an *N*-protected amino acid to form an active carboxyl, and coupling, whereby the active carboxyl reacts with the amino component to form the peptide bond.[1,8] Recently, an alternative pathway to enhance coupling efficiency by modifying the carbon skeleton of uronium salts has been reported.[9] Later, the incorporation of a hydrogen-bond acceptor in the iminium part was studied, showing superiority to those described earlier.[10] Oxygen in the iminium moiety confers the reagent more solubility, enhances coupling yields, and decreases racemization, thereby allowing the use of only 1 equiv. of base. Here we report a new class of uronium salts, *N*-[(2,2-dimethyl-4,6-dioxo-1,3-dioxan-5-ylideneamino-oxy)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate (HTMU, **6**), 1-[1-(2,2-dimethyl-4,6-dioxo-1,3-dioxan-5-ylideneaminooxy)dimethylaminomorpholinomethylene]methanaminium hexafluorophosphate (HMMU, **7**), and 1-{[1-(2,2-dimethyl-4,6-dioxo-1,3-dioxan-5-ylideneaminooxy)-dimethylamino-pyrrolidinomethylene]}methanaminium hexafluorophosphate (HDmPyMU, **8**), which represent the combination of distinct carbon skeletons, such as a morpholonium-based iminium moiety and

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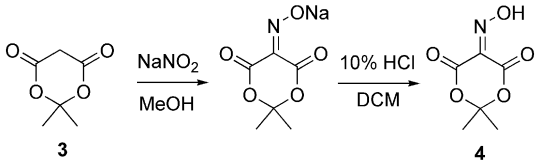
isonitroso Meldrum's acid (HONM) (**4**) as a leaving group. This new class proved to be superior coupling reagents for amide bond formation.

Results and Discussion

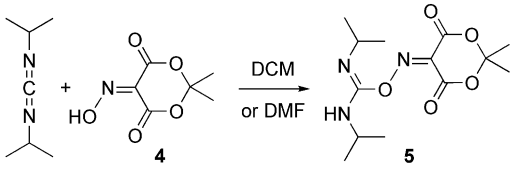
Oxyma (**2**) is an excellent replacement for HOBt (**1a**) and its analogues in terms of low racemization and high coupling efficiency.[5] With the idea to develop even more efficient coupling methods, the isonitroso Meldrum's acid HONM (**4**) was examined. HONM shows structural similarities to **2**, except for the presence of two carbonyl groups as electron-withdrawing substituents contained in the six-membered cyclic structure. This modification should enhance the reactivity of the oxime-based additive as a result of its more powerful electron-withdrawing effect compared to **2**. The cyclic structure of **4** may also be beneficial because the hydroxy function is more accessible.

HONM (**4**) was prepared by following a reported method[11] that consists of reaction of Meldrum's acid (**3**) with $NaNO_2$ in MeOH, followed by acidification with 10% HCl to afford the desired compound as an off-white solid in 69% yield (Scheme 1).



Scheme 1. Synthesis of isonitroso Meldrum's acid HONM (**4**).

The first results using **4** as additive in a *N*,*N*-diisopropylcarbodiimide (DIC)-mediated coupling were disappointing. Thus, model peptides (see below for their structure in the racemization section) were obtained with very low yields when compared with the use of **2** or benzotriazole derivatives. Given this unexpected result, we studied the reaction in depth. Thus, it was observed that the main product obtained was adduct **5**, which resulted from the side reaction of DIC and **4** (Scheme 2). The structure of this byproduct was established after following the reaction of **4** with DIC in DCM by NMR spectroscopy. Given that **2** and benzotriazole derivatives do not react with carbodiimides, the formation of adduct **5** in excellent yield indicates that **4** is highly reactive and therefore an excellent leaving group for amide formation.



Scheme 2. Reaction of isonitroso Meldrum's acid (**4**) with DIC.

Thus, HTMU (**6**), HMMU (**7**), and HDmPyMU (**8**), the HONM-based uronium salts, were prepared and tested for peptide synthesis and compared with other uronium/aminium (**9**–**14**) salts currently used in our lab and elsewhere (Figure 2).

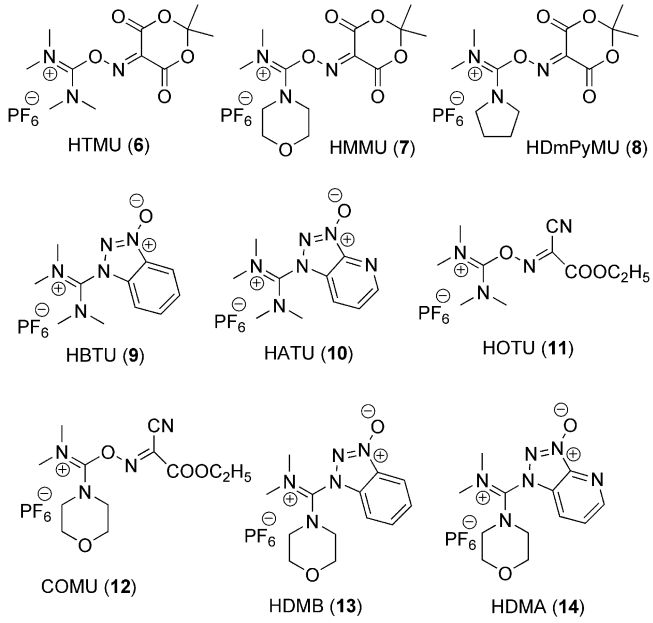


Figure 2. Structure of the coupling reagents used in the present work.

HTMU (**6**) is the tetramethyl derivative and forms part of the group of original Knorr reagents[7] such as *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU, **9**),[12] *N*-[(dimethylamino)-1*H*-1,2,3-triazolo(4,5-*b*)pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU, **10**),[3,13] and *O*-[cyano(ethoxycarbonyl)methylidene]amino-1,1,3,3-tetramethyluronium hexafluorophosphate (HOTU, **11**).[14] HMMU (**7**) incorporates a morpholino group as a hydrogen-bond acceptor in the uronium part, similarly to 1-{[1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)dimethylaminomorpholinomethylene]}methanaminium hexafluorophosphate (COMU, **12**), 1-[(dimethylamino)(morpholino)methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide (HDMB, **13**), and 1-[(dimethylamino)(morpholino)methylene]-1*H*-[1,3]triazolo-[4,5-*b*]pyridinium hexafluorophosphate 3-oxide (HDMA, **14**). These reagents have shown superiority to those described previously, as the oxygen in the uronium/iminium skeleton confers the reagent greater solubility, enhances coupling yields and decreases racemization, thereby allowing the use of only 1 equiv. of base.[10,15] With the idea to enhance the reactivity, HDmPyMU (**8**) contains a dimethylpyrrolydinium-type carbon skeleton, a modified version of the classical tetramethyl uronium salts.[9]

HONM-based uronium salts (**6**–**8**) were readily prepared following a well-established method by treating *N*,*N*-dimethylcarbamoyl chloride (**15**) with a secondary amine such as pyrrolidine or morpholine to give the corresponding

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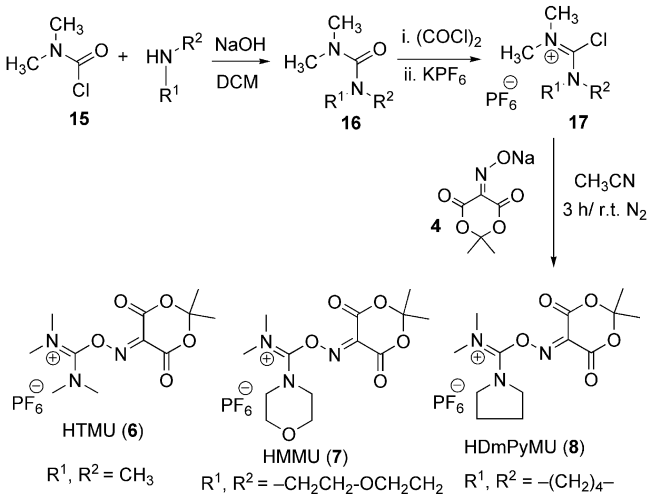
urea derivatives **16** (Scheme 3).[15] These derivatives were then treated with oxalyl chloride to yield the corresponding chloride salts, which were stabilized by the formation of a PF_6 salt (**17**). Subsequent reaction with the sodium salt of **4** under an atmosphere of N_2 in CH_3CN at room temperature afforded desired compounds **6**–**8** as crystalline and stable solids (Scheme 3).



Scheme 3. Preparation of uronium-type coupling reagents derived from HONM (**4**).

In order to determine the compatibility of HONM-based coupling reagents with automated peptide synthesizers, their stability in solution and in the solid state was examined by NMR spectroscopy by means of integrating the methyl group related to the corresponding urea (δ = 2.89 ppm) and the methyl group related to the uronium salt (δ = 3.3 ppm). The salts were stable at room temperature for 2–3 d in acetone, but after 7 d some hydrolysis was detected. The dimethylmorpholino uronium salt HMMU (**7**) was less stable than the corresponding tetramethyl derivative HTMU (**6**) (14 vs. 7% hydrolysis), whereas the dimethylpyrrolidino analogue HDmPyMU (**8**) displayed intermediate stability (13%). All reagents showed stability greater than 90% in CH_3CN in a closed vial for 3–4 d, which represents lower stability than the Oxyma-based dimethylmorpholino analogue COMU (**12**, 95%). This observation indicates that the new reagents may be more reactive than the latter.

These observations are of practical relevance for both solid-phase and solution strategies. Thus, when the activation of a carboxylic acid is slow and the coupling reagent is not stable, it is degraded and no longer able to activate the carboxylic function. This feature is crucial for cyclization steps or for segment couplings in convergent strategies, where excess amounts of the carboxylic function is not present (cyclization) or is low (segment coupling) and therefore couplings are very slow. A further characteristic of HONM-based uronium salts **6**–**8** is that the course of reaction can be followed as a result of a color change. Once the reaction is complete, the solution becomes colorless or yellow (more intense color than Oxyma derivatives, in some cases violet-like), depending on the type of base used.

To study the reactivity of these compounds, their conversion to the corresponding active esters was examined. For this purpose, *N*-benzyloxycarbonyl α-aminoisobutyric acid (Z-Aib-OH; Z = benzyloxycarbonyl, Aib = α-aminoisobutyric acid) was preactivated with the coupling reagents HMMU (**7**), COMU (**12**), HDMB (**13**), or HDMA (**14**) for 3 min in the presence of diisopropylethylamine (DIEA, 2 equiv.) in DMF at room temperature. The sample was then diluted and injected into a reverse-phase HPLC apparatus. For all the reagents tested, complete conversion to the active ester was observed after 2 min. Furthermore, the HPLC analysis clearly showed the stability of the active ester in the presence of base. After 30 min, only the active esters derived from Oxyma (**2**) and HOBt (**1a**) were observed, whereas only trace amounts from the active ester of HONM (**4**) and none of the HOAt (**1b**) ester were detected. Therefore, the relative stability of the active esters in the presence of base can be ranked in the following order: Oxyma (**2**) ≈ OBt (**1a**) > ONM (**4**) > OAt (**1b**). This order thus indicates that the cyclic oxime **4** is more reactive than **2** and similar to **1b**.

As a first model to examine the reactivity of the active esters, the hindered acid Z-Aib-OH was mixed with *p*-chloroaniline (PCA) in the presence of the corresponding coupling reagent and DIEA (1 equiv.) in DMF. Approximate extents of formation were determined by HPLC analysis on the basis of the disappearance of the active ester (Z-Aib-Oxyma **18**, Z-Aib-ONM **19**, Z-Aib-OBt **20**, and Z-Aib·OBt **21**) and the appearance of the anilide product (Z-Aib-PCA, **22**) (Scheme 4).

Table 1. Extent of formation of Z-Aib-4-chloroanilide over time by the reaction of Z-Aib-OH with 4-chloroaniline in the presence of DIEA.

Coupling reagent	Z-Aib-4-chloroanilide [%][a]								
	5 min	10 min	20 min	30 min	60 min	2 h	3 h	5 h	24 h
COMU (**12**)	19.2	28.8	47.7	65.0	75.1	85.0	89.0	93.0	98.0
HMMU (**7**)	35.8	50.0	78.5	91.0	93.3	95.4	96.0	97.0	98.0
HDMA (**14**)	17.5	28.4	64.6	71.4	78.7	86.2	87.0	93.0	96.0
HDMB (**13**)	<1	<1	<1	<1	1.0	1–2	1–2	3.0	15

[a] A true sample of the corresponding active ester was prepared by reaction of Z-Aib-Cl (0.125 mmol) with HOBt (**1a**), HOAt (**1b**), Oxyma (**2**), HONM (**4**), and 4-chloroaniline. Half-times of formation: $t_{1/2}$ (COMU, **12**) = 23 min; $t_{1/2}$ (HMMU, **7**) = 10 min; $t_{1/2}$ (HDMA, **14**) = 16 min; $t_{1/2}$ (HDMB, **13**) > 24 h. From the literature: $t_{1/2}$ (HBTU, **9** + 2 equiv. DIEA) = 11 h; $t_{1/2}$ (HATU, **10**) = 35–40 min.[16]

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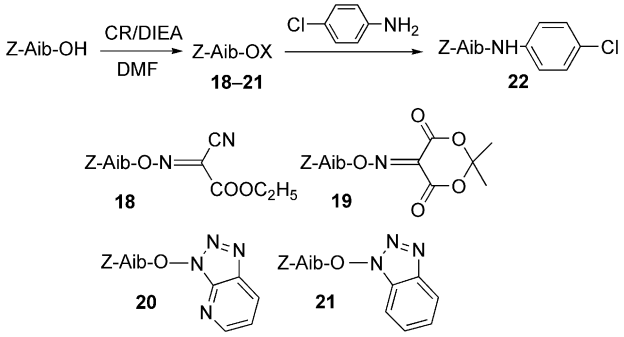

Scheme 4. Activation and coupling of Z-Aib-OH with *p*-chloroaniline (PCA) by using various coupling reagents.

The results are shown in Table 1. As expected, the HOBt-based HDMB (**13**) was the least reactive reagent, affording only 15% of the anilide in 24 h, whereas in the same time COMU (**12**), HMMU (**7**), and HDMA (**14**) yielded the desired product in an almost quantitative manner. The formation of the anilide was faster with HMMU (**7**), followed by HDMA (**14**) and COMU (**12**), as extracted from the half-times (see Footnote of Table 1).

The formation rates of the product are shown in Figure 3. HONM-based uronium salt **7** gave a high yield (>90%) of chloroanilide faster than the rest of the onium salts.

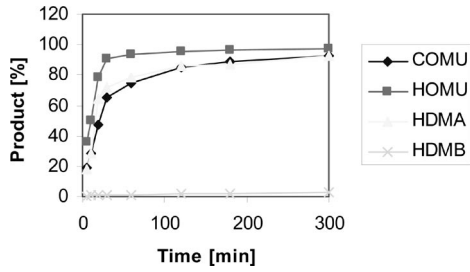

Figure 3. Extent of formation of Z-Aib-4-chloroanilide in the presence of various coupling reagents.

Table 2. Effect of coupling reagent and base on the preservation of configuration during the formation of Z-Phg-Pro-NH_2 and Z-Phe-Val-Pro-NH_2 through [1+1] and [2+1] coupling in solution, respectively.

Entry	Peptide model	Coupling reagent	Base (equiv.)	Yield [%]	DL/LDL [%]
1	Z-Phg-Pro-NH_2	HATU (**10**)	DIEA (2)[a]	78	3.1
2			TMP (2)[a]	78	2.1
3		HBTU (**9**)	DIEA (2)[a]	80	8.2
4			TMP (2)[a]	81	6.4
5		HOTU (**11**)	DIEA (2)[b]	79	0.17
6			TMP (2)[b]	90	1.20
7		COMU (**12**)	DIEA (2)[b]	88	0.12
8			TMP (2)[b]	91	0.90
9		HMMU (**7**)	DIEA (2)	89	0.2
10			TMP (2)	89	0.29
11		HTMU (**6**)	DIEA (2)	91	0.53
12			TMP (2)	89	0.33
13		HDmPyMU (**8**)	DIEA (2)	88	0.34
14			TMP (2)	87	0.50
15		HONM (**4**)/DIC[c]	–	79	0.24
16		HONM (**4**)/DIC[d]	–	80	1.27
17	Z-Phe-Val-Pro-NH_2	HATU (**10**)	DIEA (2)[a]	86	13.9
18			TMP (2)[a]	78	5.3
19			TMP (1)[b]	76	4.9
20		HBTU (**9**)	DIEA (2)[a]	90	27.7
21			TMP (2)[a]	81	14.2
22		HOTU (**11**)	DIEA (2)[b]	91	23.6
23			TMP (2)[b]	89	7.4
24			TMP (1)[b]	80	7.5
25		COMU (**12**)	DIEA (2)[b]	91	19.3
26			DIEA (1)[c]	89	8.2
27			TMP (2)[b]	90	7.0
28			TMP (1)[b]	90	3.5
29		HMMU (**7**)	DIEA (2)	90	27.8
30			DIEA (1)[c]	87	3.6
31			TMP (2)	89	5.3
32			TMP (1)	90	2.3
33		HTMU (**6**)	DIEA (2)	88	26.4
34			DIEA (1)[c]	86	10.5
35			TMP (2)	89	8.3
36			TMP (1)	83	5.5
37		HDmPyMU (**8**)	DIEA (2)	90	31.1
38			TMP (2)	89	5.4
39		HONM (**4**)/DIC[c]	–	80	14.9

[a] Previous results from ref.[10] [b] Previous results from ref.[15] [c] In these cases, 15–20% of the acid was unreacted, as indicated by HPLC analysis. Other side peaks were also observed. [d] The coupling was performed by using a 2-min preactivation period.

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Having obtained these encouraging results with HMMU (**7**), we undertook a more in-depth study using two previously reported peptide systems in order to examine the configuration retention induced by the new coupling reagents in both solution and solid phase. The novel uronium coupling reagents were tested and compared with other dimethylmorpholino [HDMA (**14**) and COMU (**12**)] and tetramethyl [HBTU (**9**), HATU (**10**), and HOTU (**11**)] analogues using these models, which involve [1+1] stepwise and also [2+1] segment coupling: Z-Phg-Pro-NH_2 and Z-Phe-Val-Pro-NH_2.[16–18]

Regarding the stepwise coupling of Z-Phg-OH to H-Pro-NH_2 (Phg = α-phenylglycine) in solution to render Z-Phg-Pro-NH_2, Oxyma-based HOTU (**11**) and COMU (**12**) and HONM-based HTMU (**6**) and HMMU (**7**) showed a greater conservation of chirality (Table 2, Entries 5, 7, 11, and 9, respectively) over the benzotriazole-based derivatives HBTU (**9**) and HATU (**10**) in the presence of DIEA (Table 2, Entries 1 and 3, respectively). Concerning the effect of the iminium moiety, the dimethylmorpholino reagents **12** and **7** induced lower racemization than tetramethyl analogues **11** and **6** (Table 2, Entries 7 and 9 vs. 5 and 11, respectively), thus confirming the positive effect of the morpholino moiety. Additive **4** (Table 2, Entry 15) showed low racemization, although as mentioned above its use is not recommended because of its high reactivity. With regard to the effect of the base used, in this stepwise model DIEA generally induced higher control of optical purity than collidine.

In the segment coupling of Z-Phe-Val-OH to H-Pro-NH_2 to afford Z-Phe-Val-Pro-NH_2, **7** gave the best results when 1 equiv. of base was used (Table 2, Entry 32). Exceptionally good results in terms of racemization and yield (2.3 and 90%, respectively) were obtained with 2,4,6-trimethylpyridine [TMP (a.k.a., collidine), 1 equiv.], whereas **6** showed an inferior performance (5.5 and 83%, respectively; Table 2, Entry 36). In contrast, when 2 equiv. of DIEA were used, **10** performed better than any other coupling reagent (Table 2, Entry 17). Consistently with previous studies, collidine induced higher control of optical purity in the fragment model than DIEA, unlike the stepwise system.[16]

Racemization during the [2+1] assembly of Z-Phe-Val-Pro-NH_2 was also tested in the solid phase by using only 1 equiv. of TMP (Table 3). Under these conditions, dimethylmorpholino uronium salts showed higher yield and lower racemization than the tetramethyl analogues (Table 3, Entries 2, 5, and 7 vs. 1, 3, 4, and 6) and again HMMU (**7**) rendered better results than the other novel HONM-based derivatives (Table 3, Entry 2 vs. 1), performing at a similar level as HOAt analogues (Table 3, Entries 3 and 5), which were the most efficient.

To demonstrate the suitability and efficiency of the novel coupling reagents in the elongation of sterically hindered sequences, HTMU (**6**), HMMU (**7**), and HDmPyMU (**8**) were compared with Oxyma- and benzotriazole-based aminium/uronium salts in the assembly of the Aib-enkephalin pentapeptide (H-Tyr-Aib-Aib-Phe-Leu-NH_2) in the solid phase by using a Fmoc/*tert*-butyl protection scheme. For this purpose, Fmoc-RinkAmide-AM-PS (0.59 mmol g^{-1}) resin was used as the solid support. Peptide elongation was performed manually by means of 30-min coupling times (with the exception of Aib-Aib coupling, where a 30 min-double coupling was applied) and 2 equiv. of DIEA with respect to coupling reagent and amino acids, the minimum amount of base in order to accomplish effective couplings, unless dimethylmorpholino salts are employed.[13] HPLC-PDA analysis indicated that Oxyma-based uronium salts were more efficient than HONM counterparts in this demanding system (Table 4, Entries 5 and 6 vs. 7–9, respectively), and COMU (**12**) showed better performance than the rest of the coupling reagents (Table 4, Entry 6). HMMU (**7**), which also rendered a high percentage of pentapeptide, was more effective than the benzotriazole derivatives HATU (**10**), HBTU (**9**), and HDMB (**13**) (Table 4, Entry 7 vs. 1, 2, and 4). Dimethylmorpholino aminium/uronium salts performed at a superior level than their tetramethyl or dimethylpyrrolidino counterparts, and HMMU (**7**) was the

Table 3. Effect of coupling reagent on the preservation of coupling during the formation of Z-Phe-Val-Pro-NH_2 through [2+1] coupling in the solid phase.

Entry	Coupling reagent	Base (equiv.)	Yield [%]	LDL [%]
1	HTMU (**6**)	TMP (1)	90	17.4
2	HMMU (**7**)	TMP (1)	91	15.1
3	HATU (**10**)	TMP (1)[a]	90	13.0
4	HBTU (**9**)	TMP (1)[a]	89	27.0
5	HDMA (**14**)	TMP (1)[a]	90	12.0
6	HOTU (**11**)	TMP (1)[a]	85	25.0
7	COMU (**12**)	TMP (1)[a]	92	21.0

[a] Previous results from ref.[15]

Table 4. The percentage of des-Aib (4-mer) (H-Tyr-Aib-Phe-Leu-NH_2) during solid-phase assembly of pentapeptide (H-Tyr-Aib-Aib-Phe-Leu-NH_2).[a]

Entry	Coupling reagent	Base (equiv.)	Penta [%]	des-Aib [%]
1	HATU (**10**)	DIEA (2)[b]	83.0	17.0
2	HBTU (**9**)	DIEA (2)[b]	47.0	53.0
3	HDMA (**14**)	DIEA (2)[b]	98.0	2.0
4	HDMB (**13**)	DIEA (2)[b]	89.0	11.0
5	HOTU (**11**)	DIEA (2)[c]	99.0	1.0
6	COMU (**12**)	DIEA (2)[c]	99.7	0.3
7	HMMU (**7**)	DIEA (2)	96.0	4.0
8	HTMU (**6**)	DIEA (2)	92.6	7.4
9	HDmPyMU (**8**)	DIEA (2)	85.7	14.3
10	HONM (**4**)/DIC	–	7.5	92.5

[a] Tetrapeptide (des-Aib) was confirmed by peak overlap in the presence of an authentic sample. The crude H-Tyr-Aib-Aib-Phe-Leu-NH_2 (in yield between 80–90%) was analyzed by HPLC by using a Sun fire C_{18} (4.6 × 150 mm, 5 μm) column, with a linear gradient of 0 to 100% of 0.036% trifluoroacetic acid (TFA) in CH_3CN/0.045%TFA in H_2O over 15 min; flow rate = 1.0 mL min^{-1}, detection at 220 nm: t_R = 6.68 (pentapeptide), 6.78 (des-Aib) min. HPLC–MS showed the expected mass for the pentapeptide at *m*/*z* = 611.0, and also for des-Aib at *m*/*z* = 526. Neither des-Tyr (*m*/*z* = 448) nor tripeptide des-Aib,Tyr (*m*/*z* = 363) were observed. [b] Previous results from ref.[10] [c] Previous results from ref.[15]

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most efficient of the HONM-based coupling reagents (Table 4, Entry 7). Again, HONM (**4**) provided insufficient results (Table 4, Entry 10).

HMMU (**7**) was further tested and compared with the dimethylmorpholino aminium/uronium salts HDMA (**14**), HDMB (**13**), and COMU (**12**) in the solid-phase synthesis of a second analogue of Leu-enkephalin (H-Tyr-*N*-MeIle-*N*-MeIle-Phe-Leu-NH_2), by introducing *N*-MeIle to replace consecutive Gly residues. Peptide elongation was carried out by using the same coupling conditions as those described above. In this model peptide, which is more sterically hindered than the Aib analogue, the most effective coupling reagent was HDMA (**14**; Table 5, Entry 1), followed by COMU (**12**), which was superior to HONM-based derivative HMMU (**7**; Table 5, Entry 3 vs. 4). As observed in the previous experiments, HDMB (**13**) was the least effective of all the coupling reagents tested (Table 5, Entry 2).

The efficiency of the dimethylmorpholino coupling reagents was also tested in the solid-phase[19] elongation of a longer peptide system: the ACP (65–74) decapeptide, which contains Aib in the place of the two consecutive Ala residues (H-Val-Gln-Aib^{67}-Aib^{68}-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2). Shorter coupling times than those used in previous peptide models were applied (5 min) in order to improve our capacity to distinguish differences in efficiency (except coupling of Aib-Ile and Aib-Aib, where 30-min and 30-min double coupling, respectively, were applied). As observed

Table 5. The percentage of des-*N*MeIle (4-*mer*) (H-Tyr-*N*MeIle-Phe-Leu-NH_2) during the solid-phase assembly of pentapeptide (H-Tyr-*N*MeILe-*N*MeIle-Phe-Leu-NH_2).[a]

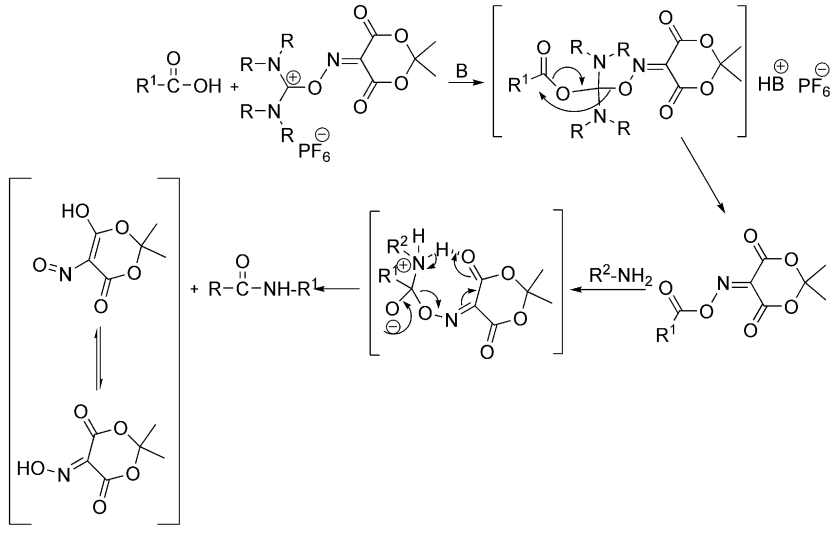
Entry	Coupling reagent	Base (equiv.)	Yield [%]	Penta [%]	des-*N*MeIle [%]	des-Tyr [%]	des-Tyr,*N*MeIle [%]
1	HDMA (**14**)	DIEA (2)	68	72.7	10.1	1.8	17.2
2	HDMB (**13**)	DIEA (2)	71	24.4	70.0	2.6	3.0
3	COMU (**12**)	DIEA (2)	70	61.2	21.7	1.2	9.1
4	HMMU (**7**)	DIEA (2)	68	44.2	24.0	2.8	8.9

[a] The ratio of the penta- and tetrapeptide was determined by reverse-phase HPLC analysis by using a Sun fire C_{18} column (5 μm, 4.6 × 150 mm), with a linear gradient of 20 to 50% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 8 min; flow rate = 1.0 mL min^{-1}, detection at 220 nm: t_R = 4.3 (pentapeptide), 2.8 (des-NMeIle), 3.5 (des-Tyr), 1.7 (des-Tyr, NMeIle) min. HPLC–MS showed the expected mass for the pentapeptide at *m*/*z* = 695.4, des-*N*MeIle at *m*/*z* = 568.4, des-Tyr at *m*/*z* = 532.4, and des-(Tyr, *N*MeIle) at *m*/*z* = 405.3.

Table 6. Synthesis of ACP (65–74) Aib^{67}-Aib^{68} analogue (H-Val-Gln-Aib-Aib-Ile^{69}-Asp-Tyr-Ile^{72}-Asn-Gly-NH_2) in the solid phase.[a,b]

Entry	Coupling reagent	Yield [%]	Deca [%]	des-Aib^{67} [%]	des-Asn [%]	des-Gln [%]	des-Ile^{69}-Ile^{72} [%]	des-Ile^{69} [%]
1	HDMA (**14**)	82	60.0	16.1	8.1	3.8	1.6	10.5
2	HDMB (**13**)	80	17.4	48.7	1.0	2.5	9.8	20.5
3	COMU (**12**)	80	80.0	<1	13.5	3.6	<1	4.3
4	HMMU (**7**)	81	76.0	<1	18.6	2.3	1.1	5.8

[a] HATU (4 equiv.) with 30 min coupling gave 50% of des-Aib^{67}, whereas HBTU afforded 90% of this tetrapeptide.[19] [b] The ratio of peaks was determined by reverse-phase HPLC analysis by using a Sun fire C_{18} column (5 μm, 4.6 × 150 mm), with a linear gradient of 10 to 35% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 8 min; flow rate = 1.0 mL min^{-1}, detection at 220 nm, t_R = 6.41 (decapeptide), 6.57 (des-Aib), 6.67 (des-Asn), 6.95 (des-Gln), 6.22 (des-Ile^{69}), 5.12 (des-Ile^{69}, Ile^{72}) min. HPLC–MS showed the expected mass for the decapeptide at *m*/*z* = 1091.2, des-Aib at *m*/*z* = 1005.1, des-Asn at *m*/*z* = 997, des-Gln at *m*/*z* = 963, des-Ile^{69} at *m*/*z* = 976.5, and des-(Ile^{69}, Ile^{72}) at *m*/*z* = 863.9.



Scheme 5. Proposed mechanism of peptide bond formation, involving a neighboring group effect by the cyclic oxime HONM (**4**).

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for the assembly of Aib-enkephalin peptide, oxime-based COMU (**12**) and HMMU (**7**) were the most effective coupling reagents (Table 6, Entries 3 and 4), as des-Aib was not detected by HPLC–MS analysis. In contrast, HDMA (**14**) and HDMB (**13**) rendered 16.1 and 48.7% of this deletion tetrapeptide, respectively (Table 6, Entries 1 and 2).

In view of the excellent results obtained with the novel uronium salts, we envisaged the existence of an additional effect that would increase their reactivity. Thus, the presence of the carbonyl groups oriented in the same direction as the *N*-*OH* group in HONM (**4**) and its derivatives can play an assisted basic catalytic role, thereby enhancing the nucleophilicity of the amine function during the coupling, as occurs when HOAt (**1b**) and *N*-ethoxycarbonyl-2-ethoxy-1,3-dihydroquinoline (EEDQ)[20] are used (Scheme 5).

Conclusions

In summary, uronium salts derived from isonitroso Meldrum's acid (HONM) are new members in the arsenal of coupling reagents. These derivatives have been shown to be very useful for acylation of nonhindered poor nucleophiles, such as *p*-chloroaniline. Furthermore, and due to their high reactivity, these derivatives provided excellent results with only 1 equiv. of base, which is a key feature for reducing racemization. However, it is important to bear in mind that HONM cannot be used as an additive for the carbodiimide, because it reacts with this functional group to form a nonreactive intermediate.

Experimental Section

General: DMF was used in peptide-grade purity. All peptides, desamino acids, and byproducts were identified by electrospray HPLC–MS. HPLC analysis was carried out with a Waters Sun fire C_{18} (5 μm, 4.6 × 150 mm) column coupled to a PDA detector. NMR spectra were recorded with a Bruker Avance 400 MHz spectrometer at room temperature. Tetramethylsilane (TMS) was used as reference for all NMR spectra, with chemical shifts reported in ppm relative to TMS. All solvents used for recrystallization, extraction, column chromatography, and TLC were of commercial grade, distilled before use, and stored under dry conditions. The model peptides for racemization experiments (Z-Phg-Pro-NH_2 and Z-Phe-Val-Pro-NH_2) were analyzed following previously described methods.[16,17]

Synthesis of Isonitroso Meldrum's Acid HONM (4):[11] A solution of $NaNO_2$ (4.14 g, 0.06 mol) in water (10 mL) was added dropwise to a solution of Meldrum's acid (7.2 g, 0.05 mol) in methanol (50 mL), with stirring (10–15 min addition). This exothermic reaction was cooled for 1.5 h in a water bath to maintain the temperature at 20–25 °C (the reaction turned pink and a precipitate was formed). The resulting precipitates were collected by suction (6.89 g, yield 68.5%) [ref.[11] 7.0 g, 71%]. Without further purification, the crude product was used for the next reaction. The sodium salt of HONM (**4**; 4.9 g, 0.25 mol) was added portion-wise to a mixture of 10% HCl (25 mL) and DCM (25 mL) with stirring. The mixture was shaken in a separatory funnel, and the DCM layer was separated. The aqueous layer was saturated with NaCl and extracted with DCM (2 × 15 mL). The combined DCM solutions were dried with anhydrous $MgSO_4$. After evaporation of the solvent, the resulting crystals (3.2 g, yield 72.7%), [ref.[11] 3.08 g, 70%] were recrystallized from DCM (20 mL) to give pale-yellow needles (m.p. 109–110 °C) [ref.[11] 109–110 °C]. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.85 (s, 6 H, 2 CH_3) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.25, 107.98, 133.94, 156.36, 157.59 ppm.

General Procedure for the Preparation of Urea Derivatives:[9] The corresponding secondary amine (0.5 mol) was dissolved in DCM (300 mL) and 10% NaOH (300 mL). Dimethyl carbamoyl chloride (0.6 mol in 200 mL of DCM) was then added over 10 min. The reaction mixture was stirred at room temperature for 3 h. The organic layer was collected, and the aqueous layer was washed with DCM (200 mL). The combined DCM solutions were washed with water (2 × 100 mL), dried with $MgSO_4$, and filtered. The solvent was removed under vacuum to give a colorless oil in 88–93% yield. The ^{1}H NMR ($CDCl_3$) spectra for the crude urea derivatives showed a high level of purity. Further purification was achieved by vacuum distillation.

***N*,*N*-Dimethylpiperidine-1-carboxamide (DmPyU):**[9,21] The pure urea was obtained as a colorless oil at 98–100 °C in 86% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.81–2.10 (m, 4 H, 2 CH_2), 2.81 (s, 6 H, 2 CH_3), 3.15–3.18 (m, 4 H, 2 CH_2) ppm.

***N*,*N*-Dimethylmorpholine-4-carboxamide (DMU):**[10] The urea derivative was distilled and collected at 127–129 °C as a colorless oil in 92% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.84 (s, 6 H, 2 CH_3), 3.22–3.2 (m, 4 H, 2 CH_2), 3.68–3.70 (m, 4 H, 2 CH_2) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 38.62, 47.51, 66.89, 164.96 ppm.

General Method for the Synthesis of Chloro–Uronium Salts:[22] Oxalyl chloride (100 mmol) in DCM (100 mL) was added dropwise to a solution of urea derivative (100 mmol) in dry DCM (200 mL) at room temperature over 5 min. The reaction mixture was then stirred under reflux for 3 h, and the solvent was removed under vacuum. The residue was washed with anhydrous ether (2 × 100 mL), and then bubbled with N_2 to remove the excess amount of ether. The crude product obtained was highly hygroscopic, so it was directly dissolved in DCM. A saturated aqueous solution of potassium hexafluorophosphate (100 mmol in 50 mL) was added at room temperature with vigorous stirring for 10–15 min. The organic layer was collected, washed once with water (100 mL), dried with anhydrous $MgSO_4$, and filtered. The solvent was removed under reduced pressure to afford a white solid, which was recrystallized from DCM/diethyl ether or CH_3CN/diethyl ether to give white crystals.

***N*-[Chloro(pyrrolidin-1-yl)methylene]-*N*-methylmethanaminium Hexafluorophosphate (DmPyCH):**[9] The product was obtained as a white solid in 89% yield; m.p. 93–95 °C. ^{1}H NMR (400 MHz, CD_3COCD_3): δ = 2.00–2.13 (m, 4 H, 2 CH_2), 3.49 (s, 6 H, 2 CH_3), 3.90–4.02 (m, 4 H, 2 CH_2) ppm.

4-[(Dimethyamino)chloromethylene]morpholin-4-iminium Hexafluorophosphate (DCMH):[10] The salt was obtained as white crystals in 90% yield (28.9 g); m.p. 94–95 °C. ^{1}H NMR (400 MHz, CD_3COCD_3): δ = 3.39 (s, 6 H, 2 CH_3), 3.75 (t, 4 H, 2 CH_2), 3.86 (t, 4 H, 2 CH_2) ppm. ^{13}C NMR (100 MHz, CD_3COCD_3): δ = 44.36, 52.82, 65.99, 162.79 ppm.

General Procedure for the Preparation of Isonitroso Meldrum's Acid–Uronium-Type Coupling Reagents: The corresponding chloride salt (20 mmol) was added to a solution of the sodium salt of HONM (20 mmol) in CH_3CN (50 mL) at 0 °C. The reaction mixture was stirred at this temperature for 30 min, allowed to reach room temperature, and then left to stir for 4 h. The crude product was filtered and washed with CH_3CN. The solvent was concen-

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trated to a small volume (1:4) under reduced pressure, and then dry ether was added to afford the product as a white solid in high purity.

***N*-[(2,2-Dimethyl-4,6-dioxo-1,3-dioxan-5-ylideneaminooxy)(dimethylamino)methylene]-*N*-methylmethanaminium Hexafluorophosphate (HTMU, 6):** The product was obtained as a white solid from CH_3CN and ether in 94% yield (3.9 g); m.p. 177–178 °C (dec.). ^1H NMR (400 MHz, [D$_6$]acetone): δ = 1.89 (s, 6 H, 2 CH_3), 3.3.37 (s, 12 H, 4 CH_3) ppm. ^{13}C NMR (100 MHz, [D$_6$]acetone): δ = 27.78, 40.71, 107.73, 141.81, 150.14, 154.75, 162.32 ppm. $C_{11}H_{18}F_6N_3O_5P$ (417.09): calcd. C 31.66, H 4.35, N 10.07; found C 31.89, H 4.47, N 10.29.

1-[1-(2,2-Dimethyl-4,6-dioxo-1,3-dioxan-5-ylideneaminooxy)dimethylaminomorpholinomethylene]methanaminium Hexafluorophosphate (HMMU, 7): The product was obtained as a white solid from CH_3CN and ether in 90% yield (4.0 g); m.p. 200–202 °C (decomp., with eff.). ^1H NMR (400 MHz, [D$_6$]acetone): δ = 1.89 (s, 6 H, 2 CH_3), 3.41 (s, 6 H, 2 CH_3), 3.82–3.84 (m, 4 H, 2 CH_2), 3.88–3.91 (m, 4 H, 2 CH_2) ppm. ^{13}C NMR (100 MHz, [D$_6$]acetone): δ = 27.8, 40.77, 49.96, 66.07, 107.80, 141.54, 150.13, 154.78, 161.48 ppm. $C_{13}H_{20}F_6N_3O_6P$ (459.10): calcd. C 34.00, H 4.39, N 9.15; found C 34.26, H 4.52, N 9.32.

1-{[1-(2,2-Dimethyl-4,6-dioxo-1,3-dioxan-5-ylideneaminooxy)-dimethylaminopyrrolidinomethylene]}methanaminium Hexafluorophosphate (HDmPyMU, 8): The product was obtained as an off-white solid from CH_3CN and ether in 96% yield (4.2 g); m.p. 164–165 °C (decomp., with eff.). ^1H NMR (400 MHz, [D$_6$]acetone): δ = 1.89 (s, 6 H, 2 CH_3), 2.06–2.11 (m, 4 H, 2 CH_2), 3.36 (s, 6 H, 2 CH_3), 3.94–3.98 (m, 4 H, 2 CH_2) ppm. ^{13}C NMR (100 MHz, [D$_6$]-acetone): δ = 25.09, 27.75, 40.39, 51.51, 107.65, 141.58, 150.13, 154.79, 159.58 ppm. $C_{13}H_{20}F_6N_3O_5P$ (443.10): calcd. C 35.22, H 4.55, N 9.48; found C 35.49, H 4.46, N 9.69.

Formation of Active Esters Using Distinct Coupling Reagent in DMF: (Z-Aib-OH) was preactivated with the coupling reagent HMMU (**11**), COMU (**13**), HDMB (**14**), or HDMA (**15**) for 3 min in the presence of DIEA (2 equiv.) in DMF at room temperature. Then, an aliquot (20 μL) of the solution was picked up and diluted to 2 mL with a mixture of CH_3CN/H_2O (1:2), and 10 μL was injected into a reverse-phase HPLC apparatus, equipped with a PDA detector, using a Sun fire C_{18} (5 μm, 4.6 × 150 mm) column, a linear gradient 0 to 100% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 8 min, flow rate = 1.0 mL min^{-1}, detection at 220 nm: t_R = 3.58 (HOAt), 3.85 (HOBt), 4.62 (HONM), 5.57 (Oxyma), 5.98 (Z-Aib-OH), 7.19 (Z-Aib-Oxyma), 7.37 (Z-Aib-ONM), 7.25 (Z-Aib-OAt), 7.84 (Z-Aib·OBt) min.

Reaction of Z-Aib-OH with 4-Chlororaniline Using Distinct Coupling Reagents in DMF: Z-Aib-OH (0.125 mmol) was mixed with the corresponding coupling reagent (0.125 mmol) and preactivated for 3 min in the presence of DIEA (2 equiv.) in DMF (2 mL) at room temperature. Then, 4-chloroaniline (0.125 mmol) was added with stirring. The resulting solution (20 μL) was picked up and diluted to 2 mL with a mixture of CH_3CN/H_2O (1:2), and 10 μL was injected into reverse-phase HPLC apparatus, equipped with a PDA detector, using a Sun fire C_{18} (5 μm, 4.6 × 150 mm) column, linear gradient 0 to 100% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 8 min, flow rate = 1.0 mL min^{-1}, detection at 220 nm: t_R = 3.58 (HOAt), 3.85 (HOBt), 4.62 (HONM), 5.57 (Oxyma), 5.98 (Z-Aib-OH), 7.19 (Z-Aib-Oxyma), 7.37 (Z-Aib-ONM), 7.25 (Z-Aib-OAt), 7.84 (Z-Aib·OBt), 3.94 (4-chloroaniline), 7.77 (Z-Aib-4-chloroanilide) min, as detected from injection of authentic samples. The reaction rate was followed by the increase in the amount of product and the decrease in the amount of active ester.

Z-Aib-4-chloroanilide:[16] An authentic sample was prepared by the reaction of Z-Aib-Cl (0.125 mmol) with 4-chloroaniline (0.125 mmol) in the presence of Et_3N (0.125 mmol) in DCM for 3 h at room temperature. The product was recrystallized from DCM/hexane to afford the product in 66% yield; m.p. 169–170 °C. ^1H NMR (400 MHz, $CDCl_3$): δ = 1.61 (s, 6 H, 2 CH_3), 5.13 (s, 2 H, CH_2), 7.08 (dd, 2 H, CH ar), 7.11–7.34 (m, 6 H, 6 CH ar), 7.45 (d, 2 H, 2 CH ar) 8.56 (br. s, 1 H, NH) ppm.

Solid-Phase Synthesis of H-Tyr-Aib-Aib-Phe-Leu-NH$_2$:[13] The synthesis was carried out in a plastic syringe, attached to a vacuum manifold so as to effect rapid removal of reagents and solvent. The Fmoc-RinkAmide-AM-PS resin, with a loading of 0.63 mmol g^{-1} (100 mg), was washed with DCM and DMF (2 × 10 mL each) and then treated with 20% piperidine in DMF (10 mL) for 10 min. The resin was then washed with DMF, DCM, and DMF (2 × 10 mL each) and acylated with a solution of Fmoc-Leu-OH (3 equiv.), the corresponding coupling reagent (3 equiv.) and DIEA (6 equiv.) in DMF (0.4 mL, previously preactivated for 1–2 min; 7-min preactivation in the case of DIC/HONM). The mixture was added to the resin and manually stirred slowly for 1 min and left to stand for 30 min (30-min double coupling only for Aib-Aib). After peptide coupling, the resin was washed with DMF and then deblocked by treatment with 20% piperidine in DMF for 7 min. The resin was washed with DMF, DCM, and DMF (2 × 10 mL each) and then coupling with the next amino acid as explained before and deblocking were repeated to obtain the pentapeptide. The peptide was cleaved from the resin with TFA/H_2O (9:1) at room temperature for 2 h. TFA was removed in vacuo, and the crude peptide was precipitated with ether. The weight of the crude peptide was recorded, and the ratio of the penta- and tetrapeptide was determined by HPLC analysis by using a Sun fire C_{18} (4.6 × 150 mm, 5 μm) column, with a linear gradient of 0 to 100% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 15 min, flow rate = 1.0 mL min^{-1}, detection at 220 nm: t_R = 6.68 (penta), 6.78 (des-Aib) min. HPLC–MS showed the expected mass for the penta at *m*/*z* = 611.0, and also for des-Aib at *m*/*z* = 526. Neither des-Tyr (*m*/*z* = 448) nor the tripeptide des-Aib,Tyr (*m*/*z* = 363) were observed.

Solid-Phase Synthesis of H-Tyr-*N*-MeIle-*N*-MeIle-Phe-Leu-NH$_2$:[15] The synthesis was carried out in a plastic syringe, attached to a vacuum manifold so as to effect rapid removal of reagents and solvent. The Fmoc-RinkAmide-AM-PS resin, with a loading of 0.7 mmol g^{-1} (100 mg) was washed with DCM and DMF (2 × 10 mL each) and then treated with 20% piperidine in DMF (10 mL) for 10 min. The resin was then washed with DMF, DCM, and DMF (2 × 10 mL each) and acylated with a solution of Fmoc-Leu-OH (3 equiv.), the corresponding coupling reagent (3 equiv.), and DIEA (6 equiv.) in DMF (0.4 mL, previously preactivated for 1–2 min). The mixture was added to the resin and manually stirred slowly for 1 min and allowed to stand for 30 min (1 h double coupling in case of *N*MeIle-*N*MeIle). After peptide coupling, the resin was washed with DMF and then deblocked by treatment with 20% piperidine in DMF for 7 min. The resin was washed with DMF, DCM, and DMF (2 × 10 mL each) and then coupling with the next amino acid as explained before and deblocking were repeated to obtain the pentapeptide. The peptide was cleaved from the resin with TFA/H_2O (9:1) at room temperature for 2 h. TFA was removed in vacuo, and the crude peptide was precipitated with ether. The precipitate was redissolved in H_2O and acetic acid (1:5) and then lyophilized. The weight of the crude peptide was recorded, and the ratio of the penta- and tetrapeptide was determined by HPLC analysis by using a Sun fire C_{18} (4.6 × 150 mm, 5 μm) column, with a linear gradient of 20 to 50% of 0.036% TFA in

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CH_3CN/0.045%TFA in H_2O over 8 min, flow rate = 1.0 mL min^{-1}, detection at 220 nm: t_R = 4.3 (pentapeptide), 2.8 (des-NMeIle), 3.5 (des-Tyr), 1.7 (des-Tyr, *N*MeIle) min. HPLC–MS showed the expected mass for the pentapeptide at *m*/*z* = 695.4, for des-*N*MeIle at *m*/*z* = 568.4, for des-Tyr at *m*/*z* = 532.4, and for des-Tyr *N*MeIle at *m*/*z* = 405.3.

Synthesis of ACP (65–74) Containing Aib [H-Val-Gln-Aib^{67}-Aib^{68}-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2]:[19] The synthesis was carried out in a plastic syringe, attached to a vacuum manifold so as to effect rapid removal of reagents and solvent. The Fmoc-RinkAmide-MBHA-PS resin, with a loading of 0.45 mmol g^{-1} (100 mg) was washed with DCM and DMF (2 × 10 mL each) and treated with 20% piperidine in DMF (10 mL) for 10 min. The resin was then washed with DMF, DCM, and DMF (2 × 10 mL each) and acylated with a solution of Fmoc-Gly-OH (2 equiv.), the corresponding coupling reagent (2 equiv.), and DIEA (2 equiv.) in DMF (0.4 mL, previously preactivated for 1–2 min). The mixture was added to the resin, followed by the addition of the second portion of DIEA (2 equiv.). The mixture was manually stirred slowly for 1 min and allowed to stand for 5 min (except coupling of Aib-Ile and Aib-Aib, where a 30-min and 30-min double coupling, respectively, was applied). After peptide coupling, the resin was washed with DMF and then deblocked by treatment with 20% piperidine in DMF for 7 min. The resin was washed with DMF, DCM, and DMF (2 × 10 mL each) and then coupling with the next amino acid as explained before and deblocking were repeated to obtain the pentapeptide. The peptide was cleaved from the resin with TFA/H_2O (9:1) at room temperature for 2 h. TFA was removed in vacuo, and the crude peptide was precipitated with ether. The precipitate was redissolved in H_2O and then lyophilized. The weight of the crude peptide was recorded, and the ratio of peaks was determined by HPLC analysis by using a Sun fire C_{18} (4.6 × 150 mm, 5 μm) column, with a linear gradient of 10 to 35% of 0.036% TFA in CH_3CN/0.045%TFA in H_2O over 8 min, flow rate = 1.0 mL min^{-1}, detection at 220 nm: t_R = 6.41 (deca), 6.57 (des-Aib), 6.67 (des-Asn), 6.95 (des-Gln), 6.22 (des-Ile^{69}), 5.12 (des-Ile^{69}, Ile^{72}) min. HPLC–MS showed the expected mass for the pentapeptide at *m*/*z* = 1091.2, des-Aib at *m*/*z* = 1005.1, des-Asn at *m*/*z* = 997, des-Gln at *m*/*z* = 963, des-Ile^{69} at *m*/*z* = 976.5, and des-(Ile^{69}, Ile^{72}) at *m*/*z* = 863.9.

Acknowledgments

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