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p-Nitrophenoxycarbonyl derivatives of Boc-protected diaminoalkanes in the synthesis of enkephalin peptidomimetics

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Abstract: The synthesis of *p*-nitrophenoxycarbonyl derivatives of 1-Boc-1,2-diaminoethane, 1-Boc-1,3-diaminopropane and 1-Boc-1,4-diaminobutane is described. These derivatives were used to synthesize five peptidomimetics, analogues of enkephalin, containing alkylurea units inside the peptide chain and at the *C*-terminal. All syntheses were carried out in solid phase on MBHA resin. Peptidomimetics with alkylurea units inserted into the peptide chain were synthesized using the standard method employing the Boc-strategy, with TFA deprotection and HF cleavage. The analogue containing a *C*-terminal alkylurea unit was synthesized using the Boc-strategy, with HCl/dioxane deprotection and TFA cleavage. All of the analogues were examined for opioid activity in GPI and MVD assays. The activity of the analogue containing a *C*-terminal alkylurea unit was comparable to that of [Leu^5]-enkephalin, while the other analogues were less active.

Keywords: *p*-nitrophenoxycarbonyl derivative; HCl deprotection; urea-based peptidomimetics

INTRODUCTION

The development of combinatorial and other solid phase techniques makes it relatively easy to discover peptidic leads, which can then be evaluated for pharmacological properties. However, peptides are rarely useful drug candidates due to their inherent limitations, including poor metabolic stability and bioavailability as well as limited oral absorption. Therefore, much attention has been devoted to transforming such leads into more stable peptidomimetics, in which non-amino acid units are incorporated into the peptide chain, in order to increase activity, resistance to enzymatic degradation as well selectivity.

In recent years, the application of the urea moiety as a replacement for the peptide bond has drawn significant interest. The hydrogen-bonding properties of the oligourea backbone [1], increased resistance to proteolytic degradation as well as promising biological properties make this class of compounds particularly suitable for drug discovery. Several reports have appeared to date, concerning the synthesis of oligoureas and urea-containing peptides in solid phase. Burgess *et al.* reported the synthesis of unnatural polymers containing repeating urea units [2] as well as the synthesis of oligoureas and enkephalin analogues [3] on the amide Rink resin, using base-labile N-phthaloyl protection of the amino group, isocyanate coupling and TFA cleavage of the product from the resin. A small library of the YGGFL-amide sequence was prepared and tested for binding to the *anti-β*-endorphin monoclonal antibody. Kim *et al.* synthesized oligoureas on the amide Rink resin [4], using optically active azido *p*-nitrophenoxycarbonyl monomers. Guichard *et al.* reported the solid phase synthesis of oligoureas on the Rink amide resin, employing activated derivatives of β^3-amino acids and using the Fmoc strategy [5]. Boeijen and Liskamp reported the synthesis of oligourea peptidomimetics on Tentagel® resin, using Boc-protected monomers [6] as well as on Argogel Rink-N-(H)-Fmoc resin, employing the Fmoc-strategy [7]. In both cases, *p*-nitrophenoxycarbonyl derivatives of mono-protected diaminoethane and amino acids have been used in the synthesis of enkephalin analogues. However, no studies assessing the biological activity of the peptidomimetics have been reported.

We recently obtained biologically active enkephalin analogues, derived from the 1–4 fragment, using the *p*-nitrophenoxycarbonyl derivative of 1-Boc-1,2-diaminoethane [8], and this encouraged us to investigate whether the application of other diaminoalkanes would yield similar results in regard to the activity of synthesized peptidomimetics. In addition, it was of interested to determine whether the presence of a *C*-terminal leucine in the analogue sequence would enhance the activity of the peptidomimetics and

Abbreviations: BDA, 1,4-diaminobutane; EDA, 1,2-diaminoethane; ESIMS, electrospray ionization mass spectrometry; PDA, 1,3-diaminopropane

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whether the attachment of an alkylurea moiety at the *C*-terminal of [Leu^5]-enkephalin would have any influence on activity.

MATERIALS AND METHODS

bis(*p*-Nitrophenyl) carbonate was purchased from Aldrich. Boc- and Fmoc-amino acids were purchased from Saxon Biochemicals Gmbh as was the MBHA resin.

General Procedure for the Synthesis of p-Nitrophenoxycarbonyl Derivatives of Boc-protected Diaminoalkanes

1-Boc-diaminoalkane (10 mmol) was added to a solution of bis(*p*-nitrophenyl) carbonate (10 mmol) in DCM (70 ml). The mixture was stirred for 2 h and then the solvent was evaporated. The residue was washed with EtOH (50 ml). Crystallization from 2-propanol (160 ml Boc-EDA, 15 ml Boc-PDA and 120 ml Boc-BDA).

p-*nitrophenoxycarbonyl derivative of Boc-EDA*. Yield, mp and analysis for *p*-nitrophenoxycarbonyl derivative of 1-Boc-1,2-diaminoethane are given in our previous paper [8].

p-*nitrophenoxycarbonyl derivative of Boc-PDA*. Yield: 34%; mp 125°–127 °C; analysis for *p*-nitrophenoxycarbonyl derivative of 1-Boc-1,3-diaminopropanone: calcd %C 53.09, %H 6.24, %N 12.38, found %C 52.98, %H 6.26, %N 12.41, H^{1}NMR (500 MHz, $CDCl_3$) δ 1.457 (s, 9H), 1.690–1.715 (m, 2H), 3.266–3.277 (m, 2H), 3.314–3.351 (m, 2H), 4.810 (s, 1H), 6.103 (s, 1H), 7.316–7.334 (d, 2H), 8.233–8.250 (d, 2H).

p-*nitrophenoxycarbonyl derivative of Boc-BDA*. Yield: 80%; mp 166°–168 °C; analysis for *p*-nitrophenoxycarbonyl derivative of 1-Boc-1,4-diaminobutane: calcd %C 54.38, %H 6.56, %N 11.89, found %C 54.43, %H 6.55, %N 11.80, H^{1}NMR (500 MHz, DMSO-d6) δ 1.380 (s, 9H), 2.503–2.511 (m, 2H), 2.914–2.951 (m, 2H), 3.064–3.101 (m, 2H), 3.332–3.358 (m, 2H), 6.811–6.833 (t, 1H), 7.3391–7.409 (d, 2H), 8.038–8.061 (t, 1H), 8.251–8.270 (d, 2H).

Synthesis of Enkephalin Peptidomimetics in Solid Phase

***Synthesis of analogues modified inside the peptide chain*.** The synthesis was carried out manually on MBHA resin (0.25 meq/g, 1% crosslink, 100–200 mesh) using Boc-amino acids and DIC as the coupling reagent (6 equiv. of the amino acid and 3 equiv. of DIC, DCM, coupling time 2.5 h, ambient temp.) and TFA deprotection, according to standard procedure. In the case of *p*-nitrophenoxycarbonyl derivatives, the amount of DMF added depended on the solubility of the respective derivative (6 equiv. of derivative, DMF, coupling time 5.5 h, 60 °C). Coupling and deprotection were monitored using the qualitative ninhydrin test. The first amino acid attached to the resin was Boc-Leu-OH, followed by Boc-Phe-OH. The resin bearing the two amino acids was then divided into four portions and respective alkylurea units were inserted instead of Gly residues (**1a** – 1 × ethylurea, **1b** – 1 × propylurea, **1c** – 1 × butylurea and **2** – 2 × ethylurea) using *p*-nitrophenoxycarbonyl derivatives of Boc-protected diaminoalkanes. Finally, Boc-Tyr[Z(2Br)]-OH was coupled. Following the removal of the Boc group, the products were cleaved from the resin with HF at 0 °C (1 h). The analogues were lyophilized and purified by HPLC.

Analysis for 1a: ESIMS: calc M 526.6 found $M + H^+$ 527.3.

Analysis for 1b: ESIMS: calc M 540.7 found $M + H^+$ 541.3.

Analysis for 1c: ESIMS: calc M 554.7 found $M + H^+$ 555.3.

Analysis for 2: ESIMS: calc M 612.7 found $M + Na^+$ 635.4.

***Synthesis of the analogue with an alkylurea moiety at the C-terminal*.** The synthesis was carried out manually on MBHA resin (0.25 meq/g, 1% crosslink, 100–200 mesh) using Boc-amino acids, Fmoc-Tyr(tBu)-OH and DIC as the coupling reagent (6 equiv. of the amino acid and 3 equiv. of DIC, DCM, coupling time 2.5 h, ambient temp.) according to standard procedure and HCl deprotection (18% HCl/dioxane, 1 × 2 min followed by 1 × 15 min, ambient temp.). Coupling and deprotection were monitored using the qualitative ninhydrin test. *p*-Nitrophenoxycarbonyl derivative of Boc-EDA was coupled to the resin (6 equiv. of derivative, DMF, coupling time: 5.5 h, 60 °C). Following deprotection four amino acids were then succesively attached: Boc-Leu-OH, Boc-Phe-OH and 2 × Boc-Gly-OH. From that point; analogue **3** was synthesized using two methods.

Method 1: Boc-Tyr[Z(2Br)]-OH was coupled and, following deprotection, the product was cleaved from the resin with HF at 0 °C (1 h). The analogue was lyophilized and purified by HPLC. ESIMS: calc M 640.7 found $M + H^+$ 641.3.

Method 2: Fmoc-Tyr(tBu)-OH was coupled and the Fmoc group was removed with 55% piperidine/DMF (1 × 20 min, followed by 1 × 30 min, ambient temp.). The product was cleaved from the resin with 55% TFA/DCM at ambient temp (15 min). The analogue was then lyophilized and the crude product was analysed using mass spectrometry. ESIMS: calc M 640.7 found $M + H^+$ 641.3 (intensity 100%) as well as impurities $M' + H^+$ 264.2 (10%) and $M'' + H^+$ 314.2 (5%).

HPLC Purification

Crude products were purified by HPLC on a Vertex Nucleosil 300 C-18 μm column (250 × 8 mm), using the following solvent system: A = 0.1% TFA/H_2O, B = 80% CH_3CN/A, with detection at 220 nm. The purity of the final products was assessed by analytical HPLC on a Vertex Eurospher 100 C-18 μm column (250 × 4.6 mm) in a linear gradient mode at a flow rate of 1 cm^3 min^{-1}, using the solvent system described above.

Mass Spectrometry

Mass spectrometry analyses were performed on a Micromass LTC (ESI-TOF) spectrometer.

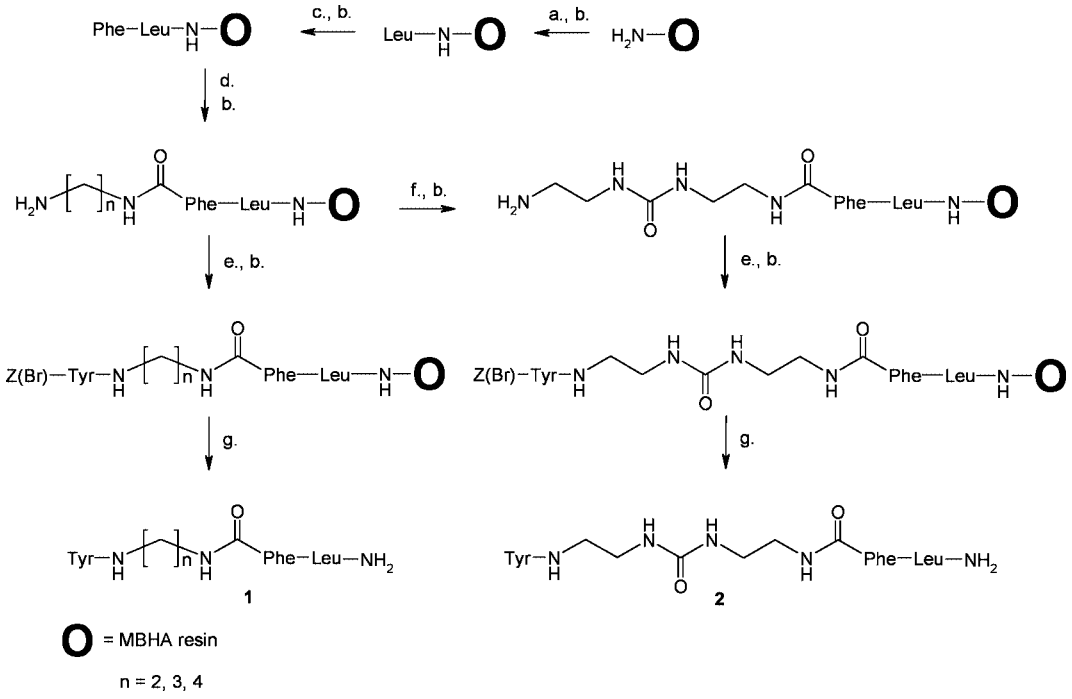
RESULTS AND DISCUSSION

Peptidomimetics were obtained recently containing alkylurea units, based on the 1–4 enkephalin sequence [8], which displayed moderate activity in MVD and GPI assays. In this work new peptidomimetics were obtained using *p*-nitrophenoxycarbonyl derivatives of Boc-protected diaminoalkanes with various lengths of the alkyl chain. The sequence of the analogues was elongated at the *C*-terminal with the Leu residue, in order to examine whether the addition of this residue would affect the activity of the peptidomimetics. An analogue was also synthesized in which an alkylurea moiety was added at the *C*-terminal of [Leu^5]-enkephalin, as it was of interest to see whether this would have any influence on the activity of the peptide.

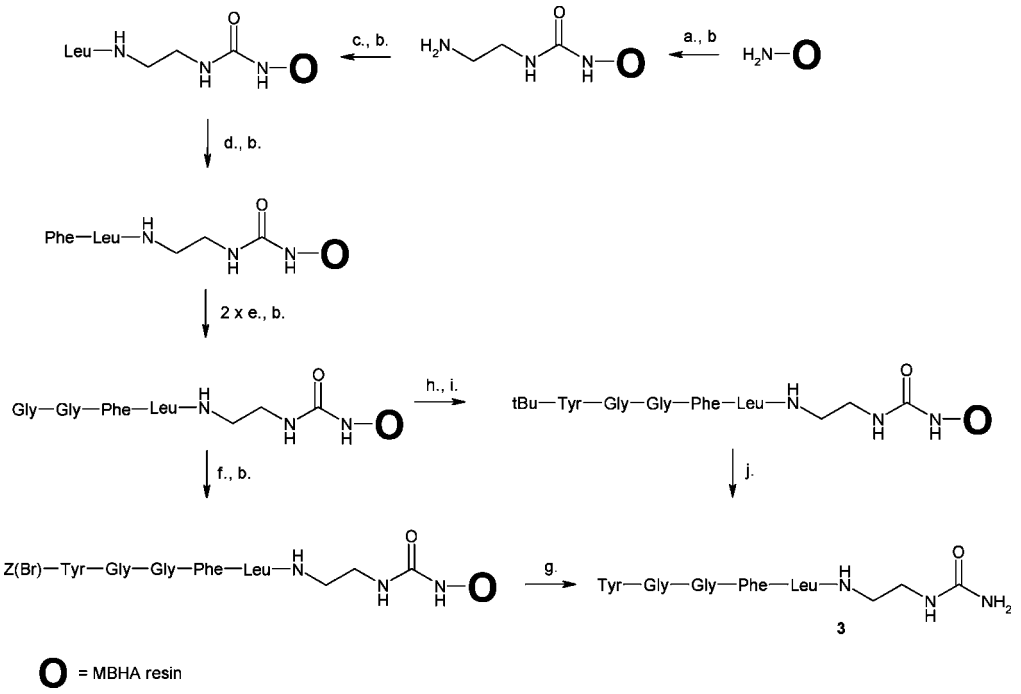
Four [Leu^5]-enkephalin analogues, in which the glycine residues were replaced with one ethylurea (**1a**), one propylurea (**1b**), one butylurea (**1c**) and two ethylurea units (**2**), were synthesized on MBHA resin using respective *p*-nitrophenoxycarbonyl derivatives of Boc-protected diaminoalkanes and Boc-amino acids (Scheme 1). The synthesis was carried out according to standard procedure, using DIC for coupling Boc-amino acids, TFA deprotection and HF cleavage.

However, a new procedure was needed for the synthesis of the analogue containing a *C*-terminal alkylurea. Peptidomimetics of similar structure were successfully synthesized using the Fmoc-protection strategy [7] on Argogel S-Ram resin, without changing the standard procedure. In our initial attempts to use MBHA resin for the synthesis of this type of peptidomimetics a poor ninhydrin reaction was observed after the first deprotection step using TFA. This prompted the use of HCl/dioxane in the synthesis of the [Leu^5]-enkephalin analogue containing a *C*-terminal ethylurea unit (**3**) (Scheme 2). This deprotection reagent had been used previously with success, in the synthesis of oligoalkylureas [8]. The synthesis was carried out along two synthetic routes. In the first method, the tyrosine residue was introduced using Boc-Tyr[Z(2Br)]-OH, while in the second, Fmoc-Tyr(tBu)-OH was used. In the former case, HF was used for cleavage and side-protection removal, while in the latter, the peptide resin was treated with 55% TFA in DCM for 15 min, which resulted in the complete cleavage of the analogue from the resin. In both cases, the yield of the crude products was comparable to that obtained for the peptidomimetics modified inside the peptide chain and synthesized using the standard procedure. Therefore, it can be concluded that the urea-MBHA linkage remains intact during deprotection carried out with the use of 18% HCl/dioxane. The purity of the obtained peptidomimetics was high, with only a marginal amount of impurities being observed in the MS spectrum of the crude product.

The obtained peptidomimetics were purified by HPLC and examined for opioid activity in the guinea-pig ileum (GPI) and mouse vas deferens (MVD) assays (Table 1). The results obtained in the biological activity assay indicate that the addition of ethylurea at the



Scheme 1 Synthesis of analogues with alkylurea units inside the chain. **a**. Boc-Leu-OH, DIC, DCM, ambient temp., 2.5 h; **b**. 55% TFA/DCM, ambient temp., 15 min; **c**. Boc-Phe-OH, DIC, DCM, ambient temp., 2.5 h; **d**. *p*-nitrophenoxycarbonyl derivative of Boc-protected diaminoalkane, DMF, 60 °C, 5.5 h; **e**. Boc-Tyr[Z(2Br)]-OH, DIC, DCM, ambient temp., 2.5 h; **f**. *p*-nitrophenoxycarbonyl derivative of 1-Boc-1,2-diaminoethane, DMF, 60 °C, 5.5 h; **g**. Liquid HF, 0 °C, 1 h.



Scheme 2 Synthesis of an analogue with an alkylurea unit at the *C*-terminal. **a**. *p*-nitrophenoxycarbonyl derivative of 1-Boc-1,2-diaminoethane, DMF, 60 °C, 5.5 h; **b**. 18% HCl/dioxane, ambient temp., 15 min; **c**. Boc-Leu-OH, DIC, DCM, ambient temp., 2.5 h; **d**. Boc-Phe-OH, DIC, DCM, ambient temp., 2.5 h; **e**. Boc-Gly-OH, DIC, DCM, ambient temp., 2.5 h; **f**. Boc-Tyr[Z(2Br)]-OH, DIC, DCM, ambient temp., 2.5 h; **g**. Liquid HF, 0 °C, 1 h; **h**. Fmoc-Tyr(tBu)-OH, DIC, DCM, ambient temp., 2.5 h; **i**. 55% piperidine in DMF, ambient temp., 1 × 20 min, 1 × 30 min; **j**. 55% TFA/DCM, ambient temp., 15 min.

Table 1 Opioid Activity Assay

		GPI		MVD	
	Compound	IC_{50} (nM)[a]	Rel. potency	IC_{50} (nM)[a]	Rel. potency
1a	H-Tyr-$NHCH_2CH_2NHCO$-Phe-Leu-NH_2	2240 ± 100	0.110 ± 0.005	P.A. (38%)[b]	
1b	H-Tyr-$NHCH_2CH_2CH_2NHCO$-Phe-Leu-NH_2	4860 ± 740	0.0506 ± 0.0077	844 ± 163	0.0135 ± 0.0026
1c	H-Tyr-$NHCH_2CH_2CH_2CH_2NHCO$-Phe-Leu-NH_2	>10000	<0.0246	6950 ± 1230 (IC_{35})[c]	0.00164 ± 0.00029
2	H-Tyr-$(NHCH_2CH_2NHCO)_2$-Phe-Leu-NH_2	>10000	<0.0246	>10000	<0.00114
3	H-Tyr-Gly-Gly-Phe-Leu-$NHCH_2CH_2NHCO$-NH_2	253 ± 14	0.972 ± 0.054	12.2 ± 1.7	0.934 ± 0.130
	[Leu^5]-enkephalin	246 ± 39	1	11.4 ± 1.1	1

[a] Mean of 3–5 determinations ±SEM.
[b] Partial agonist (max. inhibition of contractions = 38%).
[c] Partial agonist (max. inhibition of contractions = 70%).

C-terminal position of the [Leu^5]-enkephalin sequence does not change the activity of the peptide, while the replacement of the Gly residues with alkylurea units reduces activity at least by an order of magnitude. Only **1b** is a full agonist both in the GPI and the MVD assays. It should be noted that the side-chains of Tyr and Phe, which are considered to be responsible for interaction with receptors, are separated by the same linear distance (number of C + N atoms) in the chain as in enkephalin. The chain in **1a** is shorter by one atom, and one atom longer in **1c**. In our previous study, the replacement of the Gly residues with 2 ethylurea units in the 1–4 enkephalin fragment, resulted in increased activity of this analogue, as compared with the analogue containing only one such unit. In this case, compound **2**, which differs from the analogue previously studied in that it contains a *C*-terminal Leu residue, is less active. This shows that not only the linear distance between the two aromatic residues is critical for activity, but that other factors, such as conformation and the ability

to adopt the shape needed for interaction with the receptor, may be equally important.

CONCLUSIONS

Five enkephalin analogues, containing alkylurea units, were synthesized on MBHA resin. The alkylurea units were introduced inside the peptide chain or at the *C*-terminal position using *p*-nitrophenoxycarbonyl derivatives of Boc-EDA, Boc-PDA and Boc-BDA. The [Leu^5]-enkephalin analogue containing a *C*-terminal ethylurea unit was synthesized using a modified procedure, where 18% HCl/dioxane was used for Boc group removal and HF or 55% TFA/DCM for cleavage. As this analogue is equipotent with enkephalin itself in *in vitro* tests, this procedure may prove useful in the synthesis of peptides more resistant to enzymatic degradation *in vivo*. In the case of analogues modified inside the peptide chain, a lower activity was observed in relation to the parent peptide.

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