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Asymmetric synthesis of Boc-*N*-methyl-*p*-benzoyl-phenylalanine. Preparation of a photoreactive antagonist of Substance P.

Philippe Karoyan, Sandrine Sagan, Gil Clodic, Solange Lavielle and Gérard Chassaing*

Laboratoire de Chimie Organique Biologique associé au CNRS, Université P. et M. Curie, UMR 7613, Case Courrier n° 182, 4, place Jussieu, 75252 Paris Cedex 05, France

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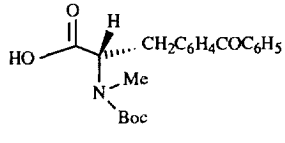
Abstract: The asymmetric synthesis of (S)-Boc-*N*-methyl-*p*-benzoyl-phenylalanine was performed by alkylation of sultam Boc-sarcosinate.The levorotatory sultam led to (S)-Boc-*N*-methyl amino acids with high optical purity. This photoreactive amino acid was incorporated into the sequence of a Substance P peptide antagonist. Comparison of the affinity and antagonistic properties of Biotinyl-apa-[D-Pro^9, MePhe(pBz)^{10}, Trp^{11}]SP for human tachykinin NK-1 receptor demonstrated that this photoreactive antagonist should be a suitable tool for photolabelling studies.

In order to probe the binding pocket for peptidic antagonists of Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) in the human NK-1 tachykinin receptor we wished to incorporate a photoreactive probe in the peptide region conferring the antagonistic activity. Ward *et al.*[1] have shown that locking the C-terminal tripeptide of Substance P in a II'β-type turn by a D-Pro-(S)-spirolactam moiety was a prerequisite for antagonistic activity. We have further established that the heterochiral dipeptides D-Pro^9-Pro^{10}, D-Pro^9-MeLeu^{10} and D-Pro^9-MePhe^{10} also restricted the conformational mobility of the C-terminal tripeptide in a II'β-type turn, yielding antagonists of Substance P: [D-Pro^9, Pro^{10}, Trp^{11}]SP, [D-Pro^9, MeLeu^{10}, Trp^{11}]SP and [D-Pro^9, MePhe^{10}, Trp^{11}]SP[2]. The introduction of an aromatic nucleus in position 10 in β-position of proline further enhanced the antagonistic activity. The benzyl-substituted prolyl analogue [D-Pro^9, Pro(β-trans-$CH_2C_6H_5$)^{10}, Trp^{11}]SP was active at the nanomolar range, being even more potent than the phenyl-substituted prolyl analogue [D-Pro^9, Pro(β-trans-C_6H_5)^{10}, Trp^{11}]SP[2]. We imagined that the incorporation of the methylated analogue of *p*-benzoylphenylalanine, Phe(pBz), in position 10 of these antagonists should lead to a photoreactive antagonist of Substance P suitable for photolabelling studies.

The purpose of this study was to develop an efficient synthesis of *N*-(Boc)-*N*-methyl-*p*-benzoylphenylalanine, Boc-MePhe(pBz), Scheme 1, the methylated derivative of one of the most currently used photoreactive amino acid, Phe(pBz)[3] and to determine the pharmacological properties of the resulting photoreactive peptide.



Scheme 1 : *N*-(Boc)-*N*-methyl-*p*-benzoylphenylalanine

* e.mail : chassain@ccr.jussieu.fr
Fax : + 33 1 44 27 71 50

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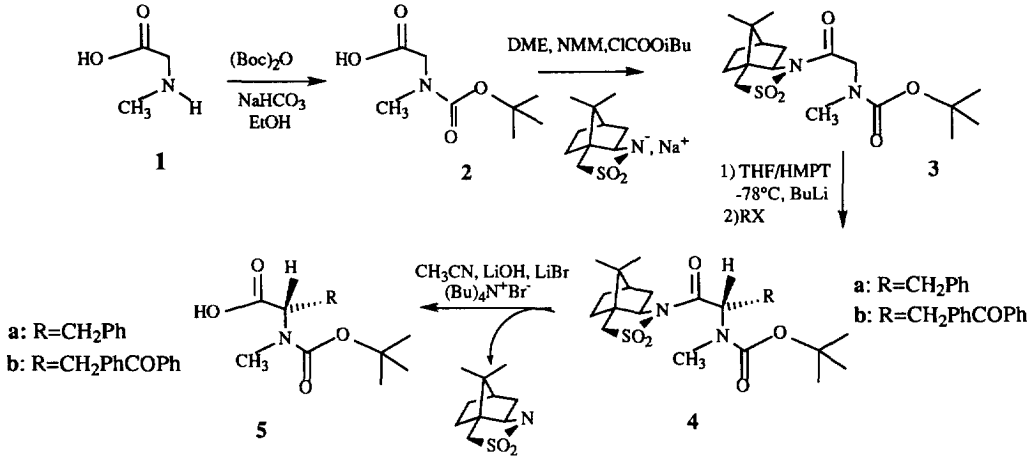
Synthesis of Boc-*N*-methyl-amino acids

We have extended Oppolzer's methodology[4,5] to the diastereoselective synthesis of non natural amino acids for structure-activity relationship and photolabelling studies[6,7]. According to this strategy, enantio pure BocPhe(pBz) may also be prepared[8]. *N*-methyl-amino acids may be prepared by different methods[9], notably by *N*-methylation with iodomethane of Boc-amino acids sodium salt[9b,c].

We have now developed a new precursor suitable for the synthesis of optically pure Boc-*N*-methyl amino acids. We wish to report this short procedure (4 steps) involving an alkylation of the chiral substrate **3** as the key step (Scheme 2). The protected *N*-methylated amino acid was directly obtained avoiding the *N*-methylation step and an eventual racemization.

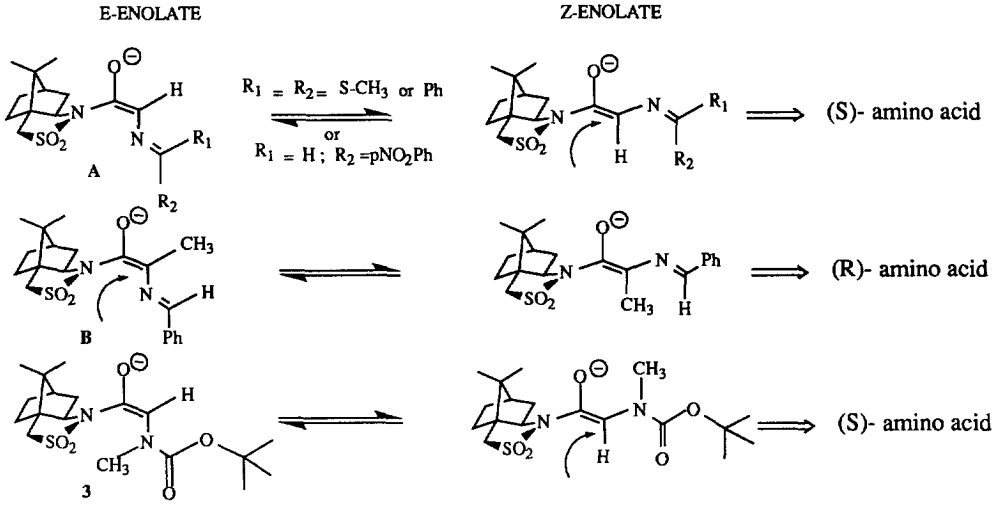


Scheme 2 : Synthesis of Boc-N-methyl amino acids

The chiral synthon **3** was obtained from sarcosine **1** after Boc protection. The Oppolzer's sultam[4] sodium salt was reacted with the carboxylic function of **2** after activation with isobutyl chloroformate. Alkylation of the lithiated chiral precursor was performed in THF/HMPT. NMR data showed that the alkylation by benzyl bromide or *p*-benzoyl-benzyl bromide was highly diastereoselective (> 99%). Final cleavage of the sultam moiety leading to **5** was performed by phase transfer catalysis in acetonitrile and the sultam was recovered. The absolute configuration of the α-carbon was determined on compound **5a** by comparison of optical rotation with a commercial sample of (S)-Boc-*N*-methyl-phenylalanine. The levorotatory enantiomer of sultam yielded (S)-Boc-*N*-methyl-phenylalanine **5a** and conversely (+)-sultam led to (R)-Boc-*N*-methyl-phenylalanine[10].

On Scheme 3, are listed the different synthons containing the sultam as a chiral auxiliary, taking into consideration the enolic position: $COC(\alpha)H_2NH$ for **A**, $COC(\alpha)HCH_3NH$ for **B**; and $COC(\alpha)H_2NCH_3$ for **3**. An interesting feature with these precursors is that the levorotatory sultam did not lead to the same absolute configuration at the α-carbon after C-alkylation. Oppolzer et al.[5] suggested that the topicity of the alkylation of ketimines **A** came from a kinetically controlled formation of chelated (Z)-enolates, alkylated from the C(α)-*Si*-face. The substituents on the imine function (bis(methyl)thiomethylene[4,5], diphenylmethylene[6] or para-chlorophenylmethylene[7]) did not affect the orientation of this attack. The present study shows that the introduction of a methyl group on the nitrogen (precursor **3**) led to an asymmetric induction identical to that

observed with non methylated precursors, such as A derivatives. Thus, *N*-methylation did not affect the approach of the electrophile and the alkylation must also occur at the C(α)-*Si*-face of the (Z)-enolate **3**, in contrast to C-methylated precursor for which the alkylation led to (R)-C(α) methyl amino acid[7].



Scheme 3 : Asymmetric inductions

Synthesis of the photoreactive peptide Bapa-[D-Pro[9], MePhe(pBz)[10], Trp[11]]SP

Using the chiral precursor **3**, the photoreactive amino acid **5b** was obtained after alkylation by p-benzoyl-benzylbromide (alkylation step, diastereoisomeric excess over 98%, yield 67%). Hydrolysis of the sultam group led to (S)-Boc-*N*-methyl-*p*-benzoylphenylalanine **5b**[11]. This photoreactive aminoacid was introduced into the Substance P antagonist sequence [D-Pro[9], MePhe[10], Trp[11]]SP[2] at position 10.

We have recently developed a strategy which allowed us to probe in the human tachykinin NK-1 receptor the binding site for peptidic agonist with a photoreactive analogue of Substance P, (Bapa-[Phe(pBz)[8]]SP), working on picomoles (1 to 10) amount of receptor[12]. Rapid and efficient purification from the medium of the covalent receptor-ligand complex prior to or after enzymatic or chemical degradation was ensured by the high affinity of biotine-sulfone for streptavidin coated-magnetic beads. Biotine-sulfone was introduced at the *N*-terminal position of the photoreactive peptide with aminopentanoic acid as a spacer (biotine-sulfone-aminopentanoyl- is abbreviated by Bapa). Desorption of these fragments from the beads with MALDI-TOF matrix allowed the direct determination of the molecular weight and thus the identification of the amino acid(s) of the receptor interacting with the photolabelled probe.

Coupling of heterochiral *N*-substituted amino acids such as D-Pro to L-Me-amino acid or D-Pro to L-Pro is usually slow and incomplete. Furthermore, in our case coupling and cyclization into diketopiperazine[13] might also be competitive reactions. Bapa-[D-Pro[9], MePhe(pBz)[10], Trp[11]]SP was obtained, as the initial antagonist [D-Pro[9], MePhe[10], Trp[11]]SP, that is after manual[14] coupling of the last three C-terminal residues on MBHA-resin, leading to Boc-D-Pro-Me(pBzl)-Phe-Trp(For)-MBHA-resin. The best result was obtained by activation of a large excess (x20) of Boc-D-Pro with DCC rapidly introduced onto Boc-MePhe(pBz)-Trp(For)MBHA-resin

after N-Boc-deprotection and neutralization. Next residues, including Boc-aminopentanoic acid, were then automatically coupled to Boc-D-Pro-Me(pBzl)-Phe-Trp(For)-MBHA-resin. Biotine-sulfone (2.5-fold excess) was incorporated manually[14] after an overnight coupling in *N*-methylpyrrolidone-2 and activation by DCC-HOBt. Formyl removal, HF cleavage and HPLC purification led to Bapa-[D-Pro^9, MePhe(pBz)^{10}, Trp^{11}]SP in 6% yield, starting from 0.1 mmol of resin[15].

Binding and antagonistic potencies of Bapa-[D-Pro^9, MePhe(pBz)^{10}, Trp^{11}]SP

The biological properties[16] of Bapa-[D-Pro^9, MePhe(pBz)^{10}, Trp^{11}]SP were determined on CHO cells transfected with the human tachykinin NK-1 receptor and compared with those of the initial antagonist[8] [D-Pro^9, MePhe^{10}, Trp^{11}]SP. The antagonist potencies were also compared with the best NK-1 peptidic antagonists: [D-Pro^9, Pro(β-trans-$CH_2C_6H_5$)^{10}, Trp^{11}]SP and GR 82334[1] and a non peptidic antagonist CP 96345[17].

Substance P and [Pro^9]SP were equally potent in displacing [^{3}H][Pro^9]SP from CHO-expressed human tachykinin NK-1 receptor, NKA the endogenous ligand for tachykinin NK-2 receptor being a poor competitor of [^{3}H][Pro^9]SP specific binding. In spite of these high differences in binding, the three peptides activated PI hydrolysis with great potency ($EC_{50} \approx$ 1-10 nM). However, the potency of NKA in stimulating cAMP formation was lower, corroborating binding data[16]. Concerning the antagonists, Bapa-[D-Pro^9, MePhe(pBz)^{10}, Trp^{11}]SP was the best competitor in binding experiments, being about 5 times more potent than the parent compound [D-Pro^9, MePhe^{10},Trp^{11}]SP and only 8 times less potent than the non peptide antagonist CP 96345 (Table 1).

Table 1. Comparison of affinities and activities of tachykinin agonists and antagonists in binding and functional assays on intact CHO cells expressing the human tachykinin NK-1 receptors.

Peptides	CHO/hNk-1 receptor[b]		
	K_i(nM)[c]	EC_{50}(nM) IPs	EC_{50}(nM) cAMP
SP[a]	1.6 ± 0.4	1.0 ± 0.6	8 ± 2
[Pro^9]SP[a]	1.1 ± 0.1	1.2 ± 0.3	10 ± 2
NKA	630 ± 80	8 ± 2	1240 ± 60
[D-Pro^9, MePhe^{10}, Trp^{11}]SP	110 ± 15	-	-
Bapa-[D-Pro^9, MePhe(pBz)^{10}, Trp^{11}]SP	23 ± 3	-	-
[D-Pro^9, Pro(β-trans-$CH_2C_6H_5$)^{10}, Trp^{11}]SP	79 ± 29	-	-
GR 82334	56 ± 15	-	-
CP 96345[a]	3.1 ± 0.9	-	-

a : Data taken from Sagan *et al.*[16a]; b : binding experiments and bioassays were conducted as described by Sagan *et al.*[16] ; c : radioligand [^{3}H][Pro^9]SP, competition experiments[16].

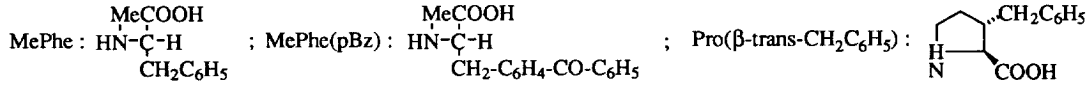
The potencies of these antagonists (pA_2 or pK_B^* values for uncompetitive antagonism) to inhibit PI hydrolysis and cAMP formation are listed in Table 2. All the peptidic antagonists were competitive antagonists on [Pro^9]SP- or NKA-induced PI hydrolysis and on [Pro^9]SP-induced cAMP formation. The pA_2 values were not significantly different whatever the second messenger or the agonist used. Interestingly in agreement with

binding data, the photoreactive antagonist Bapa-[D-Pro^9, MePhe(pBz)^{10}, Trp^{11}]SP was more potent (one order of magnitude) than the parent compound [D-Pro^9, MePhe^{10}, Trp^{11}]SP.

Table 2. Blockade of [Pro^9]SP- or NKA-induced PI hydrolysis and cyclic AMP formation by either peptidic or non peptidic (CP 96345) antagonists[a].

	IPs		cAMP
AGONISTS	[Pro^9]SP	NKA	[Pro^9]SP
ANTAGONISTS	pA_2 or pK_B*		pA_2
[D-Pro^9, MePhe^{10}, Trp^{11}]SP	6.46 ± 0.17	6.40 ± 0.18	6.94 ± 0.14
Bapa-[D-Pro^9, MePhe(pBz)^{10}, Trp^{11}]SP	7.35 ± 0.12	7.78 ± 0.12	7.19 ± 0.13
[D-Pro^9, Pro(β-trans-$CH_2C_6H_5$)^{10}, Trp^{11}]SP	7.60 ± 0.20	8.0 ± 0.1	7.74 ± 0.06
GR 82334	7.20 ± 0.20	7.9 ± 0.2	7.80 ± 0.20
CP 96345[b]	7.78 ± 0.20	8.10*[c] ± 0.30	7.81 ± 0.24

a : bioassays were conducted as described by Sagan *et al.*[16a]; b : Data taken from[16a]; c : pK_B^* for uncompetitive antagonism.



We succeeded in the design of a photoreactive peptidic antagonist of Substance P, the photosensitive reporter being part of the chromophor which imparts antagonist properties to the peptide. Therefore, Bapa-[D-Pro^9, MePhe(pBz)^{10}, Trp^{11}]SP should be a suitable tool for photolabelling studies.

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10. $[\alpha]_D^{25}$ -81 (*c* 0.5, EtOH), N-Boc-MePhe from Bachem: $[\alpha]_D^{25}$ -82 (*c* 0.5, EtOH). The asymmetric induction was further confirmed by the absolute configuration of BocMePhe(pBz) obtained with the same sultam. Indeed, Bapa-[D-Pro^9, MePhe(pBz)^{10}, Trp^{11}]SP was an efficient antagonist of Substance P, whereas the D-Pro-D-MePhe(pBz) dipeptide would have yield to a compound with very low potency (< 10^{-6}M), according to structure-activity relationship (see references 1 and 2).
11. General procedure for alkylation : BuLi (1.1 equiv.) was added under argon to an anhydrous solution of Boc-sarcosine-sultam **3** in THF/HMPT (5/1), at -78°C. The mixture was stirred for 5 min and the electrophile was added dropwise. After overnight stirring at room temperature, the mixture was quenched with CH_3CO_2H 1 equiv. in ether. After addition of ether, the organic layer was washed with aqueous saturated NH_4Cl (3x) dried over $MgSO_4$ and concentrated *in vacuo*. *N*-Boc-*N*-methyl-*p*-benzoylphenylalanine sultam **4b** : According to general procedure for alkylation. From Boc-sarcosine-sultam **3** (2 g, 5.1 mmols), n-BuLi (3.5 ml, 5.1 mmols), $PhCOPhCH_2Br$ (2.5 g, 7.6 mmol). After purification by flash chromatography (cyclohexane/ethyl-acetate, 8/2) and recrystallization (ether/pentane) white crystals were obtained (1 g, 67%) : mp : 72-74°C ; $[\alpha]_D^{25}$ -32.5 (*c*1, $CHCl_3$) ; ^{1}H-NMR (400-Mhz, $CDCl_3$) : δ 7.70-7.65 (m, 4H), 7.53-7.49 (tr, 1H), 7.41-7.31 (m, 4H), 5.73-5.69 (ABX, 1H), 3.9-3.8 (m, 1H), 3.44-3.35 (AB, 2H), 3.3-3.2 (m, 1H), 3.1-2.9 (m, 1H), 2.90-2.82 (2s, 3H), 2.1-1.78 (m, 5H), 1.45-1.35 (m, 2H), 1.35-1.28 (2s, 9H), 0.93-0.91 (2s, 3H), 0.87 (s, 3H). ^{13}C-NMR (100-Mhz, $CDCl_3$) : δ 132, 130, 129.7, 129, 128, 64.8, 52.8, 44.2, 38, 32.5, 30, 27.8, 26.7, 26.2, 20.3, 19.6. Anal. calcd for $C_{27}H_{40}N_2O_6S$: C, 66.20 ; H, 6.89 ; N, 9.82 ; found : C, 66.07 ; H, 7.04 ; N, 9.62. To a solution of *N*-Boc-*N*-methyl-*p*-benzoylphenylalanine sultam **4b** (580 mg, 1 mmole) in CH_3CN (3 ml), LiOH (146 mg, 3.5 mmol), LiBr (350 mg, 4 mmol) and Bu_4NBr (130 mg, 0.4 mmol) were added. After stirring 1 hr, water (10 ml) was added extraction with ethyl-acetate allowed sultam recovery acidification with citric acid and extraction led to **5b** ; (356 mg yield, 93 %) ; recrystallization ; mp 52-54°C $[\alpha]_D^{25}$ -57 (*c*1, $CHCl_3$) ; ^{1}H-NMR (400-MHz, $CDCl_3$) : δ 9.6-8.1 (broad peak, 1H), 7.80-7.76 (m, 4H), 7.63-7.59 (m, 1H), 7.52-7.48 (m, 2H), 7.37-7.33 (m, 2H), 4.89-4.85-4.72-4.71 (ABX, 1H, cis-trans), 3.46-3.41 (ABX, 1H), 3.28-3.13 (ABX, 1H), 2.81-2.74 (2s, 3H), 1.43-1.38 (2s, 9H). ^{13}C-NMR (100-Mhz, $CDCl_3$) : δ 176–175.4, 142.3, 137.6, 136, 132.5, 130.5, 130, 129, 128.3, 81, 61.1, 60.4, 35.4, 34.8, 33, 32.7, 28.3.Anal. calcd for $C_{22}H_{25}NO_5$: C, 66.92 ; H, 6.52 ; N, 3.65 ; found : C, 66.95 ; H, 6.54 ; N, 3.71.
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13. Manual coupling (into a reactor) allowed to check by the ninhydrin test the kinetics of the coupling reactions. In the case of the tripeptide synthesis Boc-D-Pro-MePhe(pBz)-Trp-MBHA-resin the efficiency was established by quantitative determination with picric acid, (Gisin test)[14] of the remaining amino group after deprotection of Boc-DPro onto the resin. Diketopiperazine formation was speculated since after Boc deprotection the original substitution has dropped. The best results were obtained after activation of a large excess of Boc-D-Pro with DCC. Even with such a procedure the substitution decreased from 0.68 mmol/g of resin (starting MBHA-resin) to an average of 0.35 - 0.45 mmol/g.
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15. After lyophilisation, peptides were purified by preparative reverse-phase HPLC. The separation (peaks were enlarged by cis-trans isomerism) led to 10 mg of peptide starting from 0.1 mmole of MBHA-resin (6 % yield, 98 % purity). MALDI-TOF : Bapa-[D-Pro^9, MePhe(pBz)^{10}, Trp^{11}]SP, MH^+ (monoisotopic), found: 1952.08, calcd for $C_{98}H_{130}N_{22}O_{19}S$, 1951.97
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