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$\Psi(SO_2NH)$ transition state isosteres of peptides. Synthesis and bioactivity of sulfonamido pseudopeptides related to carnosine

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Abstract

This paper reports the synthesis of tauryl dipeptides related to carnosine. In particular H-Tau-His-OH (**5**), H-Tau-His(π-Me)-OH (**6**) and H-Tau-His(τ-Me)-OH (**9**) are described. The enzyme carnosinase has been isolated from pig kidney and after purification has been used to test the stability and the inhibitory activity of the three new analogues. H-Tau-His-OH (**5**) and H-Tau-His(τ-Me)-OH (**9**) were found to possess weak inhibitory properties towards carnosinase, while H-Tau-His(π-Me)-OH (**6**) proved to be devoid of any significant activity. All the three sulfonamido pseudopeptides **5**, **6** and **9** show stability to carnosinase activity. © 1999 Elsevier Science S.A. All rights reserved.

Keywords: Carnosinase inhibitors; Carnosine; Sulfonamido peptides; Taurine; Transition state

1. Introduction

In recent years there has been an increasing interest in the structural modification of biologically active peptides in order to optimize potency, selectivity and metabolic stability [1–8].

We reported previously studies on peptides containing the residue of taurine (2-aminoethanesulfonic acid; Tau) at the *N*-terminal or internal position [9–13]. These compounds, which are characterized by the presence of a sulfonamido junction, present several interesting aspects which are connected with the biological relevance of the taurine residue as well as the stability toward enzymatic hydrolysis, the high polar character and the sulfur tetrahedral structure making it suitable for the design of tight binding enzyme inhibitors (transition state analogue protease inhibitors) [9–16]. To carry out our work in this field, we focused attention on a family of biologically relevant β-alanyl-containing dipeptides, i.e. β-alanyl-histidine (carnosine) and β-alanyl-π-methyl-histidine (anserine) (Fig. 1). These peptides occur at surprisingly high concentration in muscle and brain, the two tissues with the most active oxidative metabolism, where they have been postulated to play a role as natural antioxidants, free-radical scavengers and wound healing agents [17–20]. In particular it has been recently shown that carnosine inhibits protein alteration mediated by oxygen free-radicals and related toxic species such as hypochlorite anions and malondialdehyde [21]. However, the half-life in the body and thus the beneficial effects of these compounds are significantly limited due to the activity of the enzyme carnosinase and related peptidases which cleave the β-Ala-His [or His(Me)] peptide bond.

This report describes the synthesis and properties of the three novel pseudodipeptides tauryl-histidine (**5**), tauryl-π-methyl-histidine (**6**) and tauryl-τ-methyl-histidine (**9**), structurally related to carnosine, anserine and isoanserine [22], respectively. In contrast with the majority of previously reported sulfonamido pseudo-

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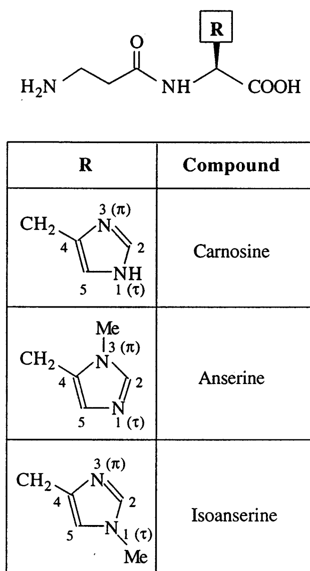


Fig. 1. β-Alanyl-containing dipeptides (numbering of the imidazole ring follows the IUPAC-IUB recommendations.

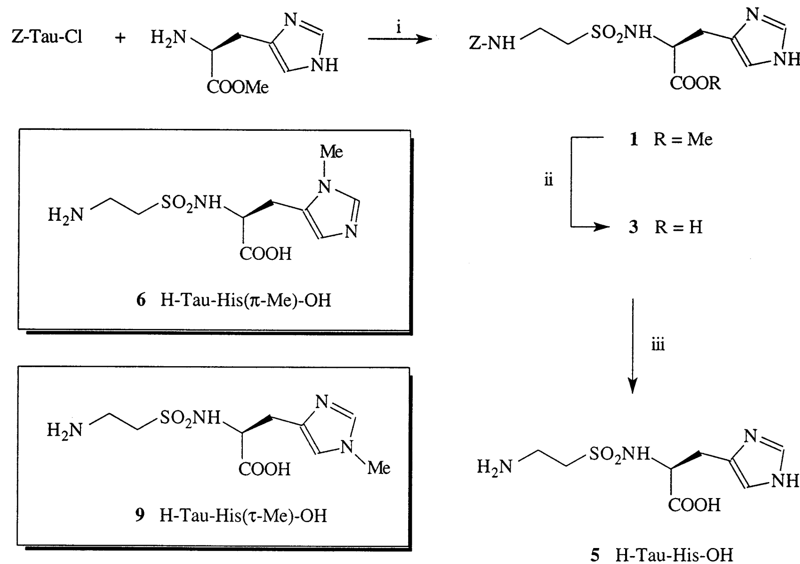
peptides, these compounds are characterized by the simple SO_2NH replacement, retaining all the other structural features of the corresponding bioactive parent. Thus, compounds **5**, **6** and **9** are very suitable models to investigate the potential of the SO_2NH junction to mimic the transition state for enzymatic amide hydrolysis [23]; in addition, they may represent therapeutically useful agents which maintain the antioxidant properties of carnosine and related dipeptides being at the same time not cleaved by serum dipeptidases [21].

2. Chemistry

Synthesis of the new carnosine analogue **5** was performed as described in Scheme 1; the same procedure has been adopted for the preparation of the anserine and isoanserine analogues **6** and **9**. As in the case of the previously studied tauryl peptides [9–13], the use of *N*-benzyloxycarbonyl chloride (Z-Tau-Cl) as acylating agent seemed a convenient synthetic route to obtain the *N*-protected tauryl dipeptide methyl esters **1**, **2** and **7**. In the case of the synthesis of Z-Tau-His-OMe (**1**), coupling of Z-Tau-Cl with equimolar amounts of H-His-OMe gave unsatisfactory results owing to the concomitant acylation at the imidazole ring N^1-position of the histidine residue. Good yields of the expected compound **1** were, however, obtained by using an excess (two equivalents) of the amino component (see Section 5). The structures of the new sulfonamido analogues **5**, **6** and **9** are in accordance with their spectroscopic properties; a correlation of the ^1H and ^{13}C NMR data with those of carnosine is reported in Table 1. In particular, the ^{13}C NMR spectrum (D_2O) of the three analogues **5**, **6** and **9** reveals that, in agreement with the different chemical environment, the Tau C^{α} signal appears shifted downfield (δ 52.44, 52.60 and 52.75, respectively) [10,11,13] as compared with the corresponding β-Ala C^{α} resonance in carnosine [24].

3. Biological results

The three analogues under study have been tested as substrates of the enzyme carnosinase and as inhibitors of the enzymatic hydrolysis of carnosine.



Scheme 1. Reagents and conditions: (i) TEA, $CHCl_3$, 0°C, 1 h, then r.t. 18 h; (ii) 1 N NaOH, (2:1) acetone–H_2O, r.t. 5 h; (iii) H_2, 10% Pd–C, (2:1) MeOH–H_2O, 3 h, r.t. Compounds **6** and **9** have been prepared by following the same procedure reported for compound **5**.

Table 1
^{1}H (300 MHz) and ^{13}C (75.43 MHz) NMR data[a] for carnosine and its sulfonamido analogues **5**, **6** and **9**

Residue	H-β-Ala-His-OH (carnosine)		H-Tau-His-OH (**5**)		H-Tau-His(π-Me)-OH (**6**)		H-Tau-His(τ-Me)-OH (**9**)	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
β-Ala or Tau								
C^{α}	2.6 m	35.09	3.05 m	52.44	3.2 m	52.60	3.2 m	52.75
C^{β}	3.2 m	38.62	3.15 m	36.84	3.35 m	37.04	3.35 m	37.10
CO		174.37						
His or His(Me)								
C^{α}	4.5 m	58.08	3.85 m	61.28	4.0 m	61.50	3.9 m	60.57
C^{β}	3.1 (4.9; 8.5; 14.6)	31.83	2.8 (4.6; 9.1; 15.1)	32.87	2.9 (4.5; 9.0; 14.7)	33.97	2.95 (5.0; 8.4; 15.4)	30.28
C^{2}	7.7 s	138.47	7.6 s	138.13	7.6 s	140.45	7.65 s	140.82
C^{4}		136.11		135.31		138.51		131.88
C^{5}	6.95 s	120.23	6.8 s	120.42	6.95 s	122.34	6.8 s	127.79
Me					3.65 s	36.23	3.55 s	34.48
CO		180.71		180.40		180.58		179.49

[a] In D_2O at 21°C. ^{13}C-chemical shifts are relative to internal dioxane. Coupling constants (in parentheses, Hz) for mutually coupled protons are given only once, at their first occurrence in the Table. The assignments for proton-bearing carbons were confirmed by APT experiments.

The sulfonamido bond turned out to be stable against hydrolytic activity of carnosinase; after 1 h incubation in the presence of 20 mM sulfonamido analogues **5**, **6** and **9**, the amount of histidine detected was less than 2% when compared with the reaction in the presence of carnosine.

H-Tau-His-OH (**5**), at a concentration of 5 mM, inhibits the hydrolysis of carnosine of about 15%. Preincubation of the enzyme for 1 h with the inhibitor does not improve the inhibition effect. H-Tau-His(τ-Me)-OH (**9**) shows a very similar behavior with about 15% inhibition under the same conditions. H-Tau-His(π-Me)-OH (**6**), however, does not inhibit the carnosinase reaction under these conditions. A detailed study of the inhibition course with H-Tau-His-OH (**5**) (data not shown) indicates the presence of a competitive inhibition pattern, with an approximate K_i value of 0.25 mM.

4. Discussion

The specificity of carnosinase involves the presence of the L-histidine residue in many of the substrates tested with this enzyme [25]. The new sulfonamido pseudopeptides described here, containing the L-histidine and related amino acids, have been found to be stable towards the activity of the serum enzyme carnosinase. Although this result would be expected by considering the high chemical stability of the SO_2NH function, it should be noted that experiments concerning the enzymatic stability of tauryl peptides towards β-alanyl specific peptidases are, at the present, not available; in fact all substrates of carnosinase referred to in the literature possess an amide or peptide bond prone to hydrolysis. Thus, he results reported here show that the analogues **5**, **6** and **9** represent interesting stable mimics of physiological components whose protective effects have been extensively reported [21].

On the other hand, data concerning the carnosinase inhibitory activity of the new products are rather unexpected and seem to confirm a recent theoretical investigation concerning the capability of phosphonamidates and sulfonamides to behave as protease transition state isosteres [23]. Two major properties of the high-energy intermediate for amide hydrolysis have been considered by the authors: the tetrahedral geometry and the electrostatic potential. The first property ensures that the inhibitor will fit into the binding site, while the second ensures that the inhibitor will interact with the enzyme in a fashion similar to the transition state [23]. Our results clearly show that the inhibition exerted by the sulfonamido carnosine analogue **5** on the carnosinase activity is not very efficient and the calculated K_i value (0.25 mM) indicates that the inhibitor has an affinity for the enzyme similar to the substrate. By considering the tetrahedral hybridization of the sulfur atom of the sulfonamides and its geometrical analogy with the amide hydrolysis transition state, it seems probable that the scarce inhibition activity shown by **5** and **9** should be attributed to an unfavorable electrostatic potential of sulfonamides as recently suggested by Houk and co-workers [23].

Regarding the complete inactivity shown by **6**, it should be noted that the substrate specificity studies with mouse kidney carnosinase [26] showed that anserine is a poor substrate for the enzyme as compared with carnosine. The π-methyl group apparently gives steric hindrance impairing the access to the bond to be hydrolyzed; H-Tau-His(π-Me)-OH (**6**) is the modified analogue of anserine and, as expected, shows only a weak inhibition.

5. Experimental

5.1. Chemistry

Melting points are uncorrected. Optical rotations were taken at 20°C with a Schmidt–Haensch Polartronic D polarimeter. IR spectra were recorded employing a Perkin–Elmer 983 spectrophotometer. ^{1}H (300 MHz) and ^{13}C (75.43 MHz) NMR spectra were determined on a Varian XL-300 instrument. ^{13}C NMR chemical shifts were measured relative to internal dioxane (67.40 ppm) for compounds **5**, **6** and **9** and relative to internal tetramethylsilane for the other compounds. Elemental analyses were performed in the laboratories of the Servizio Microanalisi del CNR, Area della Ricerca di Roma, Montelibretti, Italy and were within ±0.4% of theoretical values.

5.1.1. Preparation of Z-Tau-Xaa-OMe: general procedure

To a stirred ice-cold suspension of the corresponding 2HCl·Xaa-OMe (6.0 mmol), triethylamine (TEA) (2.4 g, 24.0 mmol) in $CHCl_3$ (5 ml) was added. After 10 min, solutions of *N*-benzyloxycarbonyltauryl chloride (3.0 mmol for compound **1**, 8.0 mmol for compounds **2** and **7**) in $CHCl_3$ (3 ml) were added dropwise at 0°C over a period of 20 min. Stirring was continued for 1 h at 0°C and 18 h at room temperature. The mixture was then filtered and the resulting solution diluted with $CHCl_3$ and washed with saturated aqueous $NaHCO_3$ and H_2O. The residue obtained after drying and evaporation was chromatographed on silica gel using $CHCl_3$–MeOH (9:1 for compound **1**; 95:5 for compounds **2** and **7**) as eluant to give the corresponding methyl esters.

Z-Tau-His-OMe (**1**). Yield 0.65 g (53%). M.p. 131–132°C (EtOAc). $[\alpha]_D = -16.0°$ ($c = 2$, EtOH). IR ν_{max} ($CHCl_3$): 3330, 3300, 3050, 1730, 1680, 1535, 1150 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ (ppm) 2.85 (2H, m, His β-CH_2), 2.95 (2H, m, Tau α-CH_2), 3.3 (2H, m, Tau β-CH_2), 3.6 (3H, s, OMe), 4.2 (1H, m, His α-CH), 5.0 (2H, s, CH_2O), 6.8 (1H, d, His C^5H), 7.3 (6H, m, ArH and Tau NH), 7.55 (1H, s, His C^2H), 7.9 (1H, d, $J = 6.5$ Hz, His NH), 11.8 (1H, broad, His $N^{\tau}H$). *Anal.* (C, H, N, S) for $C_{17}H_{22}N_4O_6S$.

Z-Tau-His(π-Me)-OMe (**2**). Yield 0.7 g (27%); oil. $[\alpha]_D = +18.0°$ ($c = 2$, $CHCl_3$). IR ν_{max} ($CHCl_3$): 3445, 3350, 1745, 1715, 1510, 1150 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ (ppm) 3.0 [2H, m, His(π-Me) β-CH_2], 3.35 (2H, m, Tau α-CH_2), 3.45 (2H, m, Tau β-CH_2), 3.6 (3H, s, $N^{\pi}Me$), 3.7 (3H, s, OMe), 4.1 [1H, m, His(π-Me) α-CH)], 5.05 (2H, s, CH_2O), 6.8 [1H, s, His(π-Me) C^5H)], 7.3 (6H, m, ArH and Tau NH), 7.55 [1H, s, His(π-Me) C^2H], 8.05 [1H, d, $J = 8.5$ Hz, His(π-Me) NH]. *Anal.* (C, H, N, S) for $C_{18}H_{24}N_4O_6S$.

Z-Tau-His(τ-Me)-OMe (**7**). Yield 1.4 g (54%). M.p. 144–145°C (EtOAc). $[\alpha]_D = +5.0°$ ($c = 2$, $CHCl_3$). IR ν_{max} ($CHCl_3$): 3265, 3180, 3110, 1720, 1690, 1150 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ (ppm) 2.85 [2H, m, His(τ-Me) β-CH_2], 2.9 (2H, m, Tau α-CH_2), 3.25 (2H, m, Tau β-CH_2), 3.55 (3H, s, $N^{\tau}Me$), 3.65 (3H, s, OMe), 4.2 [1H, m, His(τ-Me) α-CH)], 5.05 (2H, s, CH_2O), 6.9 [1H, s, His(τ-Me) C^5H)], 7.35 (6H, m, ArH and Tau NH), 7.45 [1H, s, His(τ-Me) C^2H], 7.85 [1H, d, $J = 8.2$ Hz, His(τ-Me) NH]. *Anal.* (C, H, N, S) for $C_{18}H_{24}N_4O_6S$.

5.1.2. Hydrolysis of Z-Tau-Xaa-OMe: general procedure

To a solution of the above reported methyl esters (1.3 mmol) in a mixture of (2:1) acetone–H_2O (10 ml) 1 N NaOH (2.0 ml) was added at room temperature. After 5 h at room temperature the solution was evaporated under reduced pressure and the residue taken up in H_2O. The aqueous alkaline solution was washed with CH_2Cl_2 and the pH adjusted to 5.0 with 1 N HCl. The residue obtained after evaporation was extracted with a mixture of (95:5) $CHCl_3$–MeOH to give the corresponding acids as vitreous oils.

Z-Tau-His-OH (**3**). Yield 0.5 g (96%). $[\alpha]_D = +11.0°$ ($c = 2$, EtOH). IR ν_{max} (KBr): 3400, 3150, 1705, 1600, 1150 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ (ppm) 2.9–3.0 (4H, m, His β-CH_2 and Tau α-CH_2), 3.3 (2H, m, Tau β-CH_2), 4.1 (1H, m, His α-CH), 5.0 (2H, s, CH_2O), 6.9 (1H, d, His C^5H), 7.3 (7H, m, ArH, Tau NH and His NH), 7.75 (1H, s, His C^2H). *Anal.* (C, H, N, S) for $C_{16}H_{20}N_4O_6S$.

Z-Tau-His(π-Me)-OH (**4**). Yield 0.5 g (95%). $[\alpha]_D = +7.5°$ ($c = 1$, H_2O). IR ν_{max} (KBr): 3410, 3160, 1735, 1585, 1150 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ (ppm) 2.9–3.2 [4H, m, His(π-Me) β-CH_2 and Tau α-CH_2], 3.35 (2H, m, Tau β-CH_2), 3.6 (3H, s, $N^{\pi}Me$) 4.05 [1H, m, His(π-Me) α-CH)], 5.05 (2H, s, CH_2O), 6.9 [1H, s, His(π-Me) C^5H)], 7.35 (5H, m, ArH), 7.9 [1H, s, His(π-Me) C^2H]. *Anal.* (C, H, N, S) for $C_{17}H_{22}N_4O_6S$.

Z-Tau-His(τ-Me)-OH (**8**). Yield 0.5 g (95%). $[\alpha]_D = +12.0°$ ($c = 2$, EtOH). IR ν_{max} (KBr): 3415, 3145, 1710, 1605, 1145 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm) 2.85 [2H, m, His(τ-Me) β-CH_2], 2.9 (2H, m, Tau α-CH_2), 3.25 (2H, m, Tau β-CH_2), 4.1 [1H, m, His(τ-Me) α-CH)], 5.1 (2H, s, CH_2O), 6.9 [1H, s, His(τ-Me) C^5H)], 7.4 (5H, m, ArH), 7.55 [1H, s, His(τ-Me) C^2H]. *Anal.* (C, H, N, S) for $C_{17}H_{22}N_4O_6S$.

5.1.3. Preparation of carnosine-, anserine- and isoanserine-analogues *5*, *6* and *9*: general procedure

The above described *N*-protected peptide acids (1.1 mmol) were hydrogenated in a mixture of (2:1) MeOH–H_2O (8 ml) in the presence of 10% Pd on activated charcoal (0.25 g). The mixture had been vigorously stirred for 1 h at room temperature and a further 0.1 g of catalyst was added. After additional 2 h the catalyst was filtered off and the filtrate evaporated under reduced pressure to afford the corresponding

TLC pure title compounds, further purified by crystallization.

Carnosine-analogue (**5**). Yield 0.25 g (87%). M.p. 240–242°C (EtOH–H_2O). $[\alpha]_D = -16.0°$ ($c = 2$, H_2O). IR ν_{max} (KBr): 3370, 3130, 1650, 1595, 1565, 1150 cm^{-1}. *Anal.* (C, H, N, S) for $C_8H_{14}N_4O_4S$.

Anserine-analogue (**6**). Yield 0.29 g (95%). M.p. 233–234°C (dec., EtOH–H_2O). $[\alpha]_D = -10.0°$ ($c = 2$, H_2O). IR ν_{max} (KBr): 3255, 1650, 1570, 1150 cm^{-1}. *Anal.* (C, H, N, S) for $C_9H_{16}N_4O_4S$.

Isoanserine-analogue (**9**). Yield 0.27 g (91%). M.p. 221–222°C (^{i}PrOH–H_2O). $[\alpha]_D = -11.0°$ ($c = 2$, H_2O). IR ν_{max} (KBr): 3200, 1655, 1610, 1140 cm^{-1}. *Anal.* (C, H, N, S) for $C_9H_{16}N_4O_4S$.

5.2. Enzyme assay

Carnosine and dithiothreitol (DTT) were purchased from Sigma. DEAE cellulose was a Bio-Rad product. Histidine was a Merck product. *o*-Phtaldialdehyde, Tris and trichloroacetic acid were Fluka products. The other reagents were analytical reagent grade.

The enzyme carnosinase (aminoacyl-L-hystidine hydrolase EC 3.4.13.3) [27] was obtained from pig kidney, further purified by ion-exchange chromatography on a DEAE cellulose column in phosphate buffer 0.01 M, pH 7.6. After washing, the enzyme was eluted with the same buffer containing 0.4 M NaCl. The enzyme preparation used throughout this work shows a specific activity, with 10 mM carnosine as substrate, of 0.02 mM histidine min^{-1} mg^{-1} protein without activation and of 0.05 mM min^{-1} mg^{-1} protein when the enzyme was activated with 0.01 M $MnCl_2$ (60 min preincubation in 50 mM Tris–HCl buffer, pH 8 at 25°C). The protein concentration was 6.9 mg ml^{-1}, determined by Lowry's method [28] with bovine serum albumin as standard. With carnosine as substrate, the enzyme preparation shows a K_m value of 0.5 mM which corresponds to the literature value for carnosinase of mouse [26] and hog kidney peak 1 [29]. Carnosinase activity was followed by determining the amount of histidine produced after 30 min incubation at 30°C in 50 mM Tris–HCl buffer, pH 8. The incubation mixture contained: carnosine 20 mM, enzyme 2.1 mg, 50 mM Tris–HCl buffer, pH 8, 1 mM DTT, inhibitor 0.5–5 mM, water to a final volume of 2 ml. The determination of the amount of histidine produced by the enzyme was measured using the method reported by Lenney and co-authors [30].

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