Synthesis of the Bioactive Tripeptide Bursin By Classical Methods

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O tripeptídeo biológicamente ativo Bursina (H-Lis-L-His-Gli-NH_2) foi sintetizado na forma de seu sal tri-hidrocloreto pelo método clássico de síntese de peptídeos, em solução. Os aminoácidos protegidos N^{ϵ}-tosil lisinato [MeO-Lis(Tos)-NH_2], N_{α}-benzil-oxarbonil-N_{im}-*t*-butil-oxicarbonil histidina [Cbz-His(Boc)-OH] e N-*t*-butil-oxicarbonil glicina (Boc-Gli-OH) foram acoplados pelo método do anidrido misto, seguido pela desproteção total, com um rendimento de 21%. Além dos métodos espectroscópicos e analíticos utilizados para se determinar a pureza do produto, a possibilidade de racemização foi excluída pela análise do hidrolizado volatilizado através de cromatografia gasosa, utilizando-se fases estacionárias quirais.

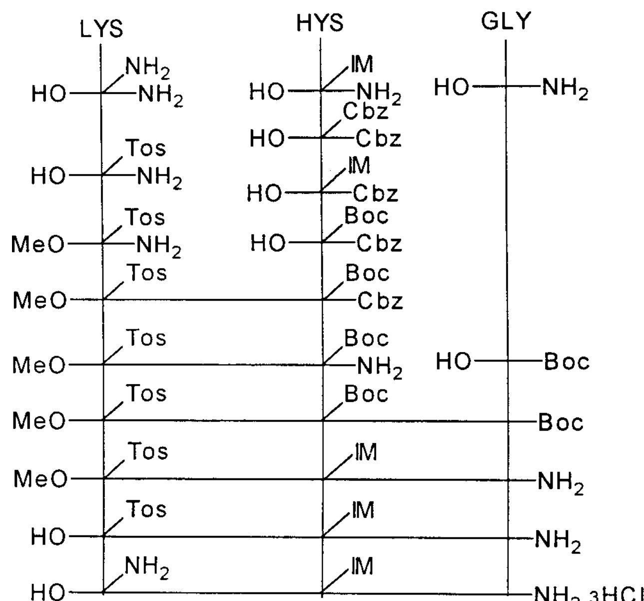
The biologically active tripeptide Bursin (H-Lys-L-His-Gly-NH_2) was wynthesized in its trihydrochloride form by classical solution based methods. Thus the protected amino acids methyl N^{ϵ}-tosyl lysinate [MeO-L-Lys(Tos)-NH_2], N_{α}-benzyloxycarbonyl-N_{im}-*tert*-butyloxy carbonyl histidine [Cbz-His(Boc)-OH] and N-*tert*-butyloxycarbonyl glycine [Boc-Gly-OH]were coupled successively in 215 total yield by the mixed anhydride method, bellowed by complete deprotection. Besides the normal spectroscopic and analytical methods for the determination of product purity, racemisation was excluded by chiral phase chromatography of volatilized hydrolysate.

Key words: *bursin; H-Lys-His-Gly-NH_2; tripeptide; mixed anhydride method.*

Introduction

The tripeptide Bursin was isolated from mammalian blood plasma[1] as well as from the bursa of Fabricius of chicken[2]. Its structure was established as H-L-Lys-L-His-Gly-NH_2. The tripeptide was shown to exhibit distinct biological activity in cell differentiation[2], tissue repair mechanisms and scar forming3 as well as collagene synthesis[4]. Bursin forms metal complexes of which the cooper(II)-Bursin complex attracted special interest because of its cell growth activity[5].

Bursin has been prepared in microscale synthesis by polymer based methods but to our knowledge so far no method suitable for the synthesis of larger quantities has emerged. Support bound methods (e.g. the Merrifield-method[6]) are expensive and difficult to perform above a certain scale. Especially for the syntheses of short peptides like Bursin, the classical solution base methods are not only a valuable alternative but still the only ones easily applicable to produce bulk quantities economically and withouth special equipment. Because of the biological significance of Bursin[7], we developed solution based synthesis of theis tripeptide. Of the two main routes followed - with and without protection of the histidine imidazole-NH-, the more successful version with full protection will be discussed here[8]. (See Scheme below)



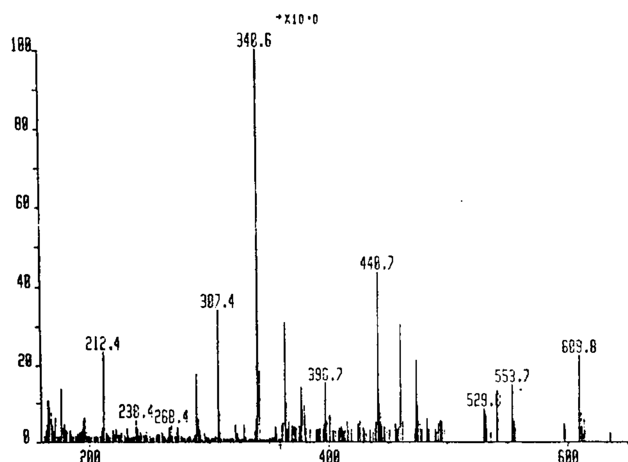


Figure 1. *Positive - FAB - spectra of* Boc-Gly-L-His(Boc)-L-Lys(Tos)-OMe *in 3-nitrobenzyl alcohol.*

Results and Discussion

For the synthesis of Bursin according to Scheme, glycine, which forms the peptide bond at the carboxyl side, was protected as *tert*-butyloxycarbonyl derivative at the amino group (Boc-Gly). The amino group of L-histidine was protected as benzoloxycarbonyl (Cbz) derivative and the imidazolic ring as *tert*-butyloxycarbonyl (Im-Boc), furnishing N_α-benzyloxycarbonyl-N_{im}-*tert*-butyloxycarbonyl-L-histid ine (Cbz-His(Boc)-OH). The side chain amino group of L-lysine was protected with the *p*-toluene sulfonyl group and the carboxyl group was converted into the methyl ester (H-Lys(Tos)OMe). The protected amino acids Cbz-His Boc)-OH and H-Lys(Tos)OMe were coupled by the mixed anhydride method using ethyl chloroformate to give the dipeptide Cbz-L-His(Boc)-L-Lys(Tos)-OMe as a yellow oil in 82% yield. The terminal amino function of this dipeptide was deprocted by catalytic hydrogenation of the Cbz-protective group with palladium on charcoal (10%) in alcoholic media, to obtain the dipeptide H-L-His(Boc)-L-Lys(Tos)-OMe as a yellow solid in 85% yield. This dipeptide was coupled with Boc-Gly by the mixed anhydride method (pivalic anhydride) to give the tripeptide Boc-Gly-L-His(Boc)-L-Lys(Tos)-OMe as a yellow solid in 61% yield. The FAB(+) spectrum (Fig. 1) of this tripeptide exhibits a protonated molecular ion peak (MH+) at m/e=709.9. Liberation of Bursin was achieved as follows: (i) deprotection of the histidine imidazolic group (Boc) with 2N HCl in dry ethyl ether, (ii) liberation of the carboxyl group of lysine by basic hydrolysis with 1N sodium hydroxide in methanol, and (iii) the tosyl group protecting the amino side chain of lysine was removed by reduction with sodium in ammonia followed by acidification with hydrochloric acid. Thus the epimerization (racemization of one amino acid residue in Bursin) was not observed, as is evidenced by the absence of diastereomeric signals in spectroscopic samples. However, the unlikely total racemization can not be excluded by normal spectroscopic methods, nor are they very sensitive. Therefore Bursin was subjected to total acidic hydrolysis (6N HCl, 110°C, 24 h), the resulting amino acid hydrochlorides were derivatized to N-Tfa-methyl esters and analyzed by GC, GC/MS and chiral GC (cyclodextrin phase). The latter showed that no racemization occured with lysine (the histidine-derivative can not be separated on the column used). Since no diastereomer is found, the synthesis proceeded without partial or total racemization, and thus allows to produce larger quantities of Bursin in a straightforward manner. Nevertheless, the yields of some steps, especially the final one, need to be improved. This is currently under investigation. Tripeptide Bursin was obtained in the trihydrochloride form as a white solid in 21% yield. The FAB(+) spectrum of Bursin (Fig. 2), exhibits a M+1 at m/e = 340.9.

Experimental

Melting points were determined on a Kofler hot plate coupled to a Reichert microscope and are uncorrected. Thin layer chromatography (TLC) was performed on precoated TLC plates (Merc, silica gel 60, F-254). The following solvent systems were used: A) $CHCl_3$-MeOH. 90:10; B) Butanol-H_2O-AcOH, 10:3:1; C) Butanol-H_2O-AcOH-pyridine, 30:24:6:20;D) Isopropanol-ammonium hydroxide, 1:1. The

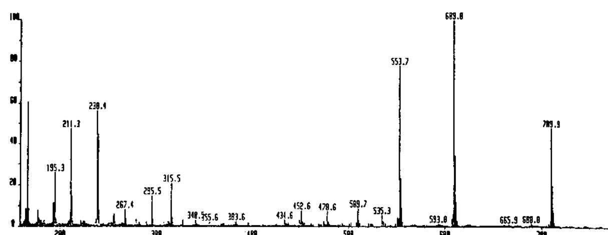


Figure 2. *Positive - FAB - spectra* Gly-L-His-L-Lys (Bursin) *in 3-nitrobenzyl alcohol.*

plates were visualized using one or more of the following methods: UV 254 nm, ninhydrin 0.1% in ethanol and otoluidine. Optical rotations were obtained on a Acatec-PBA 8300 polarimeter with a 1.0 dcm cell. Low resolution mass spectra were obtained on a GC/MS Finnigan Ion Trap Detector ITD 80A coupled to a VArian 3400 GC. FAB-MS spectra were obtained on a VG Analytical VG 70-150-S mass spectrometer equipped with a FAB ion source. The ^{1}H - and ^{13}C-NMR spectra were recorded an Bruker AC80 operating at 80.13 MHz and 20.15 MHz respectively. Gas chromatograms were recorded with a Carlo Erba HRGC-5300 gas chromatograph using a 0.32 mm capillary column of 50 m, and 0.25 mm capillary column of 25 m.

N_{α}-Benzykloxycarbonyl-N_{im}-tert-butyloxycarbonyl-L-histidyl-L-lysine(tosyl)methylester Cbz-L-His(Boc)-L-Lys(Tos)OMe). 0.79 g (2,03 mmol) CBz-His(Boc)-)H[9] were coupled with 0,71 g (2,03 mmol) L-Lys(Tos)-OMe[10] by the mixed anhydride method with ethyl chloroformate according to Lit[11] to give 1,14 g (82 %) of the coupled product as a yellow oil. ^{1}H RMN ($CDCl_3$); δ_{ppm} = 7.70 (d, 2Har); 7.28 (m, 9Har); 5.08 (s, 2H); 4.45 (t, 1H); 4.03 (t, 1H); 3.68 (s, 3H); 3.02 (d, 2H); 2,53 (m, 2H); 2.36 (3, 3H); 1.57 (s, 9H); 1.33 (m, 6H). ^{13}C RMN ($CDCl_3$) δ_{ppm} = 172.60; 171.86; 171.17; 155.75; 146.25; 142.36; 138.46; 138.10; 136.69; 127.61; 127.32; 114.53; 85.03; 66.20; 63.66; 54.20; 51.59; 42.08; 30.79; 28.29; 27.17; 21.74; 20.78.

tert-Butyloxycarbonyl-glycyl-N_{im}-tert-butyloxycarbonyl-L-histidyl-L-lysine(Tosyl) methyl ester (Boc-Gly-L-His(Boc)-L-Lys(Tos)-OMe). 0.65 g (1.18 mmol) H-=His (Boc)-L-Lys(Tos-OMe (prepared by hidrogenolysis of Cbz-L-His(Boc)-Lys(Tos)OMe according to Lit[12], p. 153) were coupled with 0.21 g (1.18 mmol) Boc-Gly[12] by the mixed anhydride method (pivaloyl chloride), in analogy to Lit[13]. 0.49 g (61%) tripeptide were obtained as an oil, which proved homogeneous on TLC in the solvet systems A and B. FAB[+][M + 1] m/e = 709.0. ^{13}C RMN ($CDCl_3$) δ_{ppm}= 172.13; 170.99; 170.55; 156.09; 148.69; 142.84; 138.20; 137.13; 129.14; 126.69; 114.95; 85.54; 80.35; 52.86; 51.74; 44.35; 42.31; 30.71; 28.16; 27.67; 21.94; 21.29.

Glycyl-L-histidyl-L-lysine(tosyl)-OH (Gly-L-LysTos)-OH). 0.2 g (0.28 mmol) of the tripeptide Boc-Gly-His(Boc)-Lys(Tos)-OMe was suspended in dry ethyl ether (10 ml) and dry hydrochloric acid gas was introduced into the suspension at room temperature for 30 minutes. The formed precipitate was collected by filtration and washed with dry ethyl ether. This product was dissolved in methanol and 1N solution of NaOH were added at room temperature according to the procedure described by Bodansky[10]. An amorphous yellow solid, homogeneous on TLC was obtained in 61.5 % yield.

Glycyl-L-histidyl-L-lysine-OHx3HCl (Gly-L-His-L-Lysx3HCl). Tlycyl-L-histidyl-L-lysine(tosyl)-OH 0.06 g (0.13 mmol) was reduced with Na/NH_3 in a procedure analogous to that given in Lit14. After acidification to pH 3 with 2N hydrochloric acid a white precipitate is formed. Washing and drying yields 21% of on amorphous white solid. R_f= 0.20 (butanol - acetic acid - pyridine - water : 30:6:20:24) R_f= 0.50 (isopropanol - ammonia hydroxide 1:1). Anal. calc. for $C_{14}H_{24}N_6O_4.H_2O$: C 34.68% H 6.55% N 20.22% H20 7.40. Found: C 34.70 H 6.60 N 19.85 H2O 7.60. FAB(+): 348.6.

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