J. prakt. Chem. 338 (1996) 380–382

Journal für praktische Chemie
Chemiker-Zeitung

Synthesis of Z-Ala-Phe-Lys(Z)-CH_3: Problem of Racemization at Cα of Lysine

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Received May 15th, 1995 respectively December 5th, 1995

Methylketones of short peptides are well known reversible inhibitors of cysteine and serine proteases. N-Acylated peptide methylketones have been synthesized by two general methods: introduction of the methylketone moiety into the final peptide or coupling the methylketone derivative with N-protected amino acids or peptides. Fittkau et al. have synthesized the methylketone peptides as inhibitors of thermitase and subtilisin [1, 2]. Mc Murray and Dyckes obtained methylketones of peptides as inhibitors of trypsin *via* a modified Dakin–West reaction [3, 4]. Complete racemization at the α-carbon of lysine was observed during conversion of Boc-Ala-Lys(Z)-OH and Boc-Lys(Z)-Ala-Lys(Z)-OH into the respective methylketones under Dakin–West conditions. Evidence for racemization was obtained from the NMR spectra of Dns-Ala-Lys(Z)-CH_3. Separation of diastereoisomers was not observed in their chromatography experiments [3].

In search of reversible, active centre directed inhibitors of plasmin and other trypsin like proteases, Z-Ala-Phe-Lys(Z)-CH_3 has been obtained. In order to develop a rational synthesis for methylketone peptides with optically active C-terminal lysine, well known methods were tested.

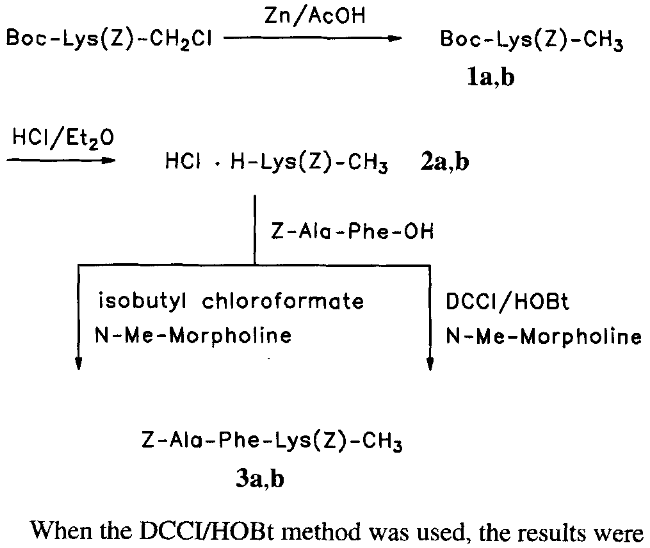
^1H NMR(^2H DMSO, 200 MHz) analysis was used to determine racemization at the α-carbon of lysine. The ketomethyl peak (integrating for three protons) of Z-*L*-Ala-*L*-Phe-*L*-Lys(Z)-CH_3 appears as a singlet at δ 1.91ppm . The same peak of Z-*L*-Ala-*L*-Phe-*D*-Lys(Z)-CH_3 appears as a singlet at δ 2.02 ppm. Therefore, determination of the ratio of diastereoisomers in crude and purified product by ^1H NMR is possible.

Results and Discussion

Reduction of chloromethylketones with zinc dust in glacial acetic acid [5] was examined. The conversion of Z-Ala-Phe-Lys(Z)–CH_2Cl to the respective tripeptide methylketone was unsatisfactory *via* this method. The reduction was not completed even after 48 hours, and chromatographic separation from unreacted substrate was not succesful.

The conversion of Boc-Lys(Z)-CH_2Cl to Boc-Lys(Z)-CH_3 was complete after 4 hours. Prolonged reaction time results in formation of by-products connected with elimination of the Boc group. N-Terminal deprotection of Boc-Lys(Z)-CH_3 under mild conditions gives optically pure HCl·H-Lys(Z)-CH_3 in 40% yield. The product can be stored under anhydrous conditions for several weeks. Longer reaction times (1h) result in the formation of partially racemized compound in 80% yield. The mixed anhydride method [5] was used to couple amino acid methylketone with *N*-protected amino acids or peptides. DCCI/HOBt coupling method was also employed in order to minimize racemization.

Synthesis of Z-L-Ala-L-Phe-L-Lys(Z)-CH_3 using the step by step procedure was unsatisfactory because of problems with *N*-terminal deprotection of dipeptidyl ketone Boc-*L*-Phe-*L*-Lys(Z)-CH_3. It was possible to avoid most of the side reactions by applying mild deprotection conditions, although the yield was low, but it was not possible to avoid racemization at Cα of lysine. The ^1H NMR spectrum of HCl·H-*L*-Phe-*L*-Lys(Z)-CH_3 reveals that two signals for methylketone protons are present: 1,92 ppm and 2.1 ppm in 3:1 ratio. When this substance was used for the synthesis of tripeptide methylketone a ratio of **3a**:**3b** was about 3:1, too. Because of this reason the 2+1 method was used to synthesize **3a** and **3b** without racemization at Cα of lysine.



When the DCCI/HOBt method was used, the results were analogous to those obtained with the the mixed anhydride method. The Dakin–West method was also used to synthesize the tripeptide methylketone from Z-*L*-Ala-*L*-Phe-*L*-Lys(Z)-OH. According to ^1H NMR analysis, the ratio of diastereoisomers was close to 1:1 in the crude product. Among purification methods, only washing with diethyl ether does not change this ratio. Crystallisation from methanol gives a product with high excess of **3a**. Chromatographic purification gave three fractions of product with different ratios of diastereoisomers. The Dakin–West method in the modification of McMurray and Dyckes is a simple and high yield method, but the ratio of diastereoisomers in the purified product is difficult to predict.

Experimental

Classical coupling techniques were used to prepare all amino acid derivatives and peptides. Melting points were taken on a Boëtius apparatus. NMR spectra were taken on Bruker AC 200 F spectrometer. Elemental analyses were performed on the Analyser CHNSO model 2400 (Perkin–Elmer). Specific rotations were measured using Polamat A (Carl Zeiss, Jena). TLC was performed on silica gel plates (Kieselgel 60 F_{254}, Merck) using following solvent systems: A – chloroform/ethanol (9:1); B – chloroform/methanol/acetic acid (17:2:1); C – chloroform/acetone (7:1). The chromatograms were stained by 1) spraying ninhydrine 2) exposing the plate to chlorine vapour for 15 min and after 20 min spraying with tolidine reagent. MN Kieselgel 60 (200–300 mesh) was used for column chromatography.

[N^{α}-(tert-Butyloxycarbonyl)-N^{ε}-(benzyloxycarbonyl)-L-lysyl]methane (Boc-L-Lys(Z)-CH_3) (**1a**)

1 g (2.4 mmol) of Boc-*L*-Lys(Z)-CH_2Cl was dissolved in 30 ml of acetic acid, and 1.5 g of zinc powder was added. After the mixture had been vigorously stirred for 4h, 70 ml of ethyl acetate were added. The solution was filtred and extracted with saturated $NaHCO_3$ and water. The organic layer was dried over $MgSO_4$. After evaporation under reduced pressure and washing with ethyl ether, a white powder was obtained. Yield 743 mg (81%), m.p. 72–73 °C, $[\alpha]_D^{20} = -1.8$ (c=1, MeOH) R_f: 0.70 (A), 0.76 (B), 0.47 (C). – ^1H NMR δ (^2H DMSO) 7.42–7.18 (m, 7H, ZC_6H_5, α NH, ε NH), 5.00 (s, 2H, ZCH_2), 3.84–3.78 (m, 1H, α CH), 3.01–2.92 (m, 2H, ε CH_2) 2.05 (s, 3H -$COCH_3$) 1.75–1.16 (m, 15H, Boc CH_3, β CH_2, γ CH_2, δ CH_2).

$C_{20}H_{30}N_2O_5$	Calcd.	C 63.47	H 7.99	N 7.40
(378.5)	Found	C 63.47	H 8.06	N 7.39

[N^{α}-(tert-Butyloxycarbonyl)-N^{ε}-(benzyloxycarbonyl)-D-lysyl]methane (Boc-D-Lys(Z)-CH_3) (**1b**)

This compound was obtained by the same procedure as for **1a**.

Yield 801mg (87.5%), m.p. 68–70 °C, $[\alpha]_D^{20} = +2.0$ (c=1, MeOH). – ^1H NMR spectra and R_f were identical to **1a**

$C_{20}H_{30}N_2O_5$	Calcd.	C 63.47	H 7.99	N 7.40
(378.5)	Found	C 63.86	H 7.92	N 7.63

[N^{α}-Benzyloxycarbonyl-L-alanyl-L-phenylalanyl-N^{ε}-(benzyloxycarbonyl)-L-lysyl] methane (Z-L-Ala-L-Phe-L-Lys(Z)-CH_3 (**3a**)

Method A (DCCI/HOBt)

200 mg (0.53 mmol) of Boc-*L*-Lys(Z)-CH_3 (**1a**) were dissolved in 10 ml of dry diethyl ether, and 5ml of dry diethyl ether saturated with HCl (2N) was added. After 15 min at room temperature the solution was quickly evaporated under reduced pressure and washed several times with dry diethyl ether. The obtained white, hygroscopic powder (yield about 40%) was dried for two hours over P_2O_5 and NaOH. After drying HCl·H-L-Lys(Z)-CH_3 (**2a**) was used without purification for the synthesis of tripeptide methylketone.

63 mg (0.2 mmol) of **2a**, Z-*L*-Ala-*L*-Phe-OH (185 mg, 0.5 mmol), HOBt (70 mg, 0.5 mmol) and 10 ml of anhydrous THF were cooled to –20 °C, then DCCI (103 mg, 0.5 mmol) and N-methylmorpholine (0.05ml, 0.5mmol) was added. The mixture was stirred at –20 °C for 1 h and for 2 h at room temperature, then it was filtered, and 40 ml of ethyl acetate was added. The solution was extracted with aq. $NaHCO_3$, 2N citric acid, $NaHCO_3$ and water, dried over $MgSO_4$ and evaporated in vacuo. The resulting product was washed with diethyl ether to give a white solid. Yield 80 mg (65%); $[\alpha]_D^{20} = -47.4$ (c=1, DMF) m. p. 162–163 °C, R_f: 0.63 (A),) 0.68 (B),) 0.14 (C). – ^1H NMR δ (^2H DMSO): 8.26 (d, 1H, Lys α NH), 8.06 (d, 1H, Phe α NH), 7.5–7.1 (m, 17H, ZC_6H_5, PheC_6H_5, Ala α NH, Lys ε NH), 4.99 (s, 4H, ZCH_2), 4.55 (m, 1H, Phe α CH), 4.15–3.95 (m, 2H, Lys α CH, Ala α CH), 3.05–2.8 (m, 4H, Lys ε CH_2, Phe CH_2), 1.91 (s, 3H, -$COCH_3$), 1.75–0.95 (m, 9H, Lys β CH_2, γ CH_2, δ CH_2, AlaCH_3).

$C_{35}H_{42}N_4O_7$	Calcd.	C 66.65	H 6.71	N 8.88
(630.7)	Found	C 65.89	H 6.74	N 8.65

Method B (mixed anhydride)

The preparation of **3a** was carried out according to the literature method [5], using **2a** obtained in the same way as in method A. Yield 100mg (42%), $[\alpha]_D^{20} = -48.6$ (c=1, DMF), m.p. 159–161 °C. – ^1H NMR δ (^2H DMSO) and R_f were identical to method A.

$C_{35}H_{42}N_4O_7$	Calcd.	C 66.65	H 6.71	N 8.88
(630.7)	Found	C 65.96	H 6.59	N 8.57

[N^{α}-Benzyloxycarbonyl-L-alanyl-L-phenylalanyl-N^{ε}-(benzyloxycarbonyl)-D-lysyl]methane (Z-L-Ala-L-Phe-D-Lys(Z)-CH_3) (**3b**)

This compound was obtained using method A described for the synthesis of **3a** (with **1b** instead of **1a**, **2b** was obtained). Yield 70 mg (55%), $[\alpha]_D^{20} = +4.0$ (c=1, DMF), m.p. 133–136°C. – R_f: 0.60 (A), 0.68 (B), 0.16 (C). – ^1H NMR δ (^2H DMSO): 8.26 (d, 1H, Lys α NH), 8.04 (d, 1H, Phe α NH), 7.5–7.1 (m, 17 H, ZC_6H_5, PheC_6H_5, Ala α NH, Lys ε NH), 4.99 (s, 4H, ZCH_2), 4.53 (m, 1H, Phe α CH), 4.15–3.95(m, 2H, Lys α CH, Ala α CH), 3.0–2.8 (m, 4H, Lys ε CH_2, PheCH_2), 2.02 (s, 3H, -$COCH_3$), 1.75–0.95 (m, 9H, Lys β CH_2, γ CH_2, γ CH_2, AlaCH_3)

$C_{35}H_{42}N_4O_7$	Calcd.	C 66.65	H 6.71	N 8.88
(630.7)	Found	C 66.09	H 6.80	N 8.71

[N^{α}-Benzyloxycarbonyl-L-alanyl-L-phenylalanyl-N^{ε}-(benzyloxycarbonyl)-DL-lysyl] methane (Z-L-Ala-L-Phe-DL-Lys(Z)-CH_3) **(3c)**

Dakin–West method

Synthesis of **3c** using Z-*L*-Ala-*L*-Phe-*L*-Lys(Z)-OH as the substrate was carried out according to the literature method [3], the **3b**:**3a** ratio was about 51.5:48.5 in the crude product. Three purification methods were tested.

Method 1

Column chromatography of the crude methylketone (102 mg) on silica gel (2% MeOH in $CHCl_3$, 2×38 cm column) gives 65 mg of the partially purified substance. This product was used to the next chromatography under the same conditions. Three fractions of the pure methylketone were obtained (total weight 54 mg (yield 49.8%). TLC suggested different ratios of diastereoisomers in each fraction. This suggestion was supported by ^1H NMR spectra and measurements of the specific rotation.

Fraction 1 (18 mg): m.p. 156–158 °C; **3b**:**3a** = 20:80; $[\alpha]_D^{20}$ = –40.9, (c=1, DMF).

Fraction 2 (20 mg): m.p. 139–148°C; **3b**:**3a** = 60:40; $[\alpha]_D^{20}$ = –21.8, (c=1, DMF).

Fraction 3 (16 mg): m.p. 130–138 °C; **3b**:**3a** = 78:22; $[\alpha]_D^{20}$ = –5.3, (c=1, DMF).

Method 2

The crude product (98 mg) was dissolved in 4 ml of hot methanol and left at room temperature for 24 hours. After filtration 17 mg (yield 16.4%) of the pure methylketone was obtained. The ratio of **3b**:**3a** was 16:84; m.p. 158–163 °C, $[\alpha]_D^{20}$ = –42.6°, (c=1, DMF).

The filtrate was evaporated in vacuum to give 78 mg of crude methylketone. The ratio of **3b**:**3a** was 64:36.

Method 3

The crude product (140 mg) was washed several times with diethyl ether to give 93 mg (yield 62.5%) of a white solid. The ratio of **3b**:**3a** was 53:47; m.p. 138–141°C, $[\alpha]_D^{20}$ = –21.8° (c=1, DMF).

$C_{35}H_{42}N_4O_7$	Calcd.	C 66.65	H 6.71	N 8.88
(630.7)	Found	C 66.2 ± 0.2	H 6.6 ± 0.1	N 8.7 ± 0.1

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