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The Synthesis of Fully Boc-Protected Insulin Derivatives by Use of Boc-Azide — 1,1,3,3-Tetramethylguanidine — 1,2,4-Triazole as an Effective Acylating System. — A Simple and Sensitive Fluorescence Test for Determining the Degree of *N*-Substitution

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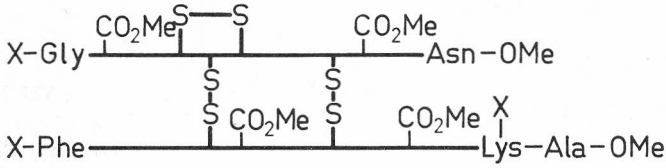
Acylation of desoctapeptide-(B^{23-30})-insulin pentamethyl ester (II), insulin and insulin hexamethyl ester was carried out with Boc-azide in dimethylformamide in the presence of 1,1,3,3-tetramethylguanidine as a base and 1,2,4-triazole as an activator to give very homogeneous di-Boc-desoctapeptide-(B^{23-30})-insulin pentamethyl ester (III), tri-Boc-insulin (IV) and tri-Boc-insulin hexamethyl ester (V), respectively, in high yield (> 90%). The degree of substitution of the products was assayed by a simple and sensitive test, based on the fluorescence reaction with primary amines, and expressed as the fluorescence intensity relative to that produced by an equimolar amount of free insulin. A comparison of the fluorescence intensities of III and IV prepared under various conditions indicates that (*a*) the Boc-azide — 1,1,3,3-tetramethylguanidine — 1,2,4-triazole combination is more effective than the Boc-azide — imidazole and Boc-azide — triethylamine acylating systems, and (*b*) the presence of 1,2,4-triazole is essential for the acylating efficiency of the system employed.

INTRODUCTION

Complete and selective protection of the amino groups in insulin and its derivatives with the *tert*-butyloxycarbonyl (Boc) groups has been often used in procedures involving specific reactions on the other parts of the peptide molecule. Levy and Carpenter[1,2] synthesized the fully protected tri-Boc-insulin (IV) by reacting insulin hydrochloride with Boc-azide in dimethylformamide in the presence of triethylamine, and the same procedure has been used[3,4] to acylate desoctapeptide-(B^{23-30})-insulin pentamethyl ester (II) into the di-Boc-derivative III. As the amino group of phenylalanine-B^1 has a greatly reduced reactivity toward Boc-azide relative to the amino group of glycine-A^1 and the ε-amino group of lysine-B^{29}, conditions were elaborated[5,6] under which the Boc group can be applied to residues A^1 and B^{29} with only a minor reaction on residue B^1. Levy[7] has shown that the extent of acylation of insulin with Boc-azide in dimethylformamide depends on the excess of reagent used, on the reaction time and on the nature of the base employed; thus, the use of imidazole instead of triethylamine markedly decreased the rate of acylation.

We report here on a modified Boc-protection procedure, involving 1,1,3,3-tetramethylguanidine as a base and 1,2,4-triazole as an activator, by which



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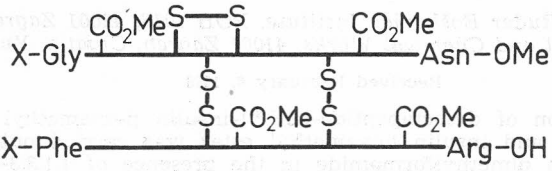

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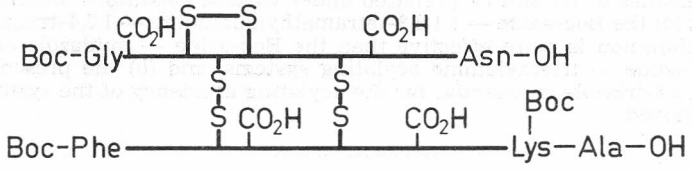

IV

very homogenous di-Boc-desoctapeptide-insulin pentamethyl ester (III), tri-Boc--insulin (IV) and tri-Boc-insulin hexamethyl ester (V), respectively, was synthesized in high yield. In order to determine the degree of substitution of the products obtained, a simple and sensitive test, based on the fluorescamine reaction[8] with primary amines, has been elaborated.

RESULTS AND DISCUSSION

Esterification of porcine insulin into the hexamethyl ester I was performed by the boron trifluoride-methanol procedure as described by Gattner et al.[9], except that we omitted the purification step on CM Sephadex column with sodium acetate — urea buffer, because of partial decomposition of the product during fractionation. In passing, it may be noted that our attempts to prepare

I by treating[3] insulin with diazomethane were also unsuccessfull; the reaction performed under various (but strictly controlled) conditions led, as already reported[4], either to formation of methylated by-products, or to incomplete esterification, or to both.

The tryptic incubation[4] of I was performed at pH 7.5 and 25 °C for 24 h, followed by precipitation of the digest at pH 8.4 and fractionation of the precipitate on a Sephadex G-50 (fine) column with 1 mol dm^{-3} acetic acid (Figure 1.). Desoctapeptide-(B^{23-30})-insulin pentamethyl ester (II) emerging in the peak *C* was completely free of the starting I, as shown by cellogel electrophoresis at pH 4.8 and by the amino acid composition of the hydrolyzate (Table I). The residue obtained in section *B* was subjected to a second fractionation to afford an additional amount (C_1) of II not contaminated with I; total yield: 47.6%.

Several improvements of the commonly used procedures for the introduction of Boc group into amino acids have been reported in the literature. Thus,

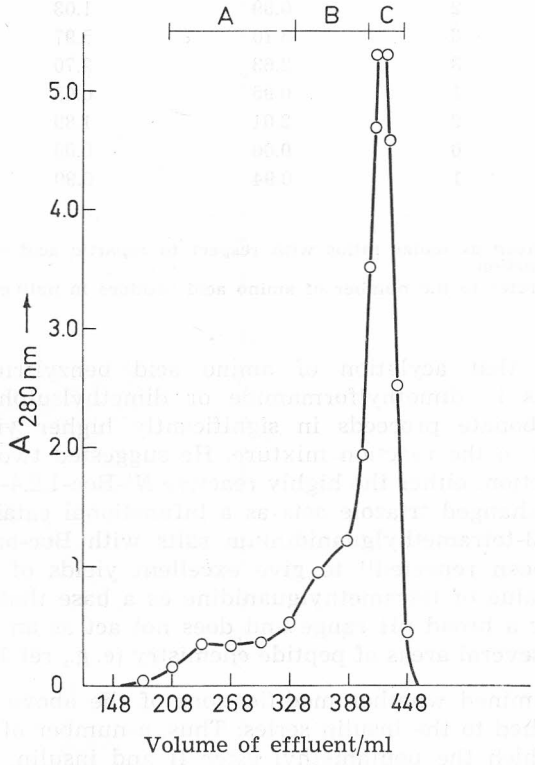

Figure 1. Gel filtration of Sephadex G-50 (fine) of the tryptic digest of I. — The precipitate derived from digestion of I (300 mg) was dissolved in 1 mol dm^{-3} AcOH (1.5 ml) and applied to a column (2.5 × 100 cm) of Sephadex G-50 (fine) which was equilibrated and developed with 1 mol dm^{-3} AcOH. Elution was performed at 15 ml/60 min, and fractions (3 ml) were pooled as indicated and lyophilized. *C* = desoctapeptide-(B^{23-30})-insulin pentamethyl ester (II), 90.7 mg. The residue left in *B* was re-run on the same column to give an additional amount (35.2 mg) of material eluting at the position of peak *C* (C_1).

TABLE I

Amino Acid Composition of Desoctapeptide-insulin Pentamethyl Ester (II) and di-Boc-Desoctapeptide-insulin Pentamethyl Ester (III)[a]

Amino Acid Residue[b]	Calc. for II and III	Found: II, from Peak *C*	Found: II, from Peak C_1	Found: III, from Expt. no. 5
Asp (3)	3	3.00	3.00	3.00
Thr (2)	1	1.12	1.00	0.89
Ser (3)	3	2.91	2.84	2.87
Glu (7)	7	7.11	7.20	6.99
Pro (1)	0	0.00	0.00	0.00
Gly (4)	3	3.04	2.87	3.01
Ala (2)	1	1.13	1.06	1.10
Cys (6)	6	4.50	3.87	4.56
Val (4)	4	2.41	2.45	2.86
Ile (2)	2	0.89	1.03	0.93
Leu (6)	6	6.10	5.97	6.00
Tyr (4)	3	2.63	2.70	2.76
Phe (3)	1	0.95	0.65	0.84
His (2)	2	2.01	1.89	1.91
Lys (1)	0	0.00	0.00	0.00
Arg (1)	1	0.94	0.90	0.98

[a] Values found are given as molar ratios with respect to aspartic acid = 3 and they are not corrected for destruction.

[b] Values in brackets refer to the number of amino acid residues in native porcine insulin.

Bram[10] observed that acylation of amino acid benzyltrimethylammonium (»Triton B«) salts in dimethylformamide or dimethylsulphoxide with *tert*-butylphenyl carbonate proceeds in significantly higher yields when 1,2,4-triazole is added to the reaction mixture. He suggested two explanations for the promoted reaction: either the highly reactive N^1-Boc-1,2,4-triazole is formed in situ or the unchanged triazole acts as a bifunctional catalyst. Acylation of amino acid 1,1,3,3-tetramethylguanidinium salts with Boc-azide in dimethylformamide has been reported[11] to give excellent yields of Boc-amino acids. In general, the value of tetramethylguanidine as a base that has no effect on racemization over a broad pH range and does not act as an acyl acceptor has been outlined in several areas of peptide chemistry (e. g., ref 12—14).

We have examined whether modifications of the above type are also effective when applied to the insulin series. Thus, a number of experiments was carried out in which the pentamethyl ester II and insulin, respectively, was treated with varying excesses of Boc-azide in dimethylformamide at controlled pH in the presence of 1,1,3,3-tetramethylguanidine, with or without addition of 1,2,4-triazole. In parallel runs, the synthesis of di-Boc-desoctapeptide-insulin pentamethyl ester (III) and tri-Boc-insulin was carried out under conditions of the existing methods[2,4,7]. We found benzyltrimethylammonium hydroxide unsuitable as a base, mainly because of its tendency to contaminate the product.

The degree of substitution of the products was assayed by fluorometric technique using fluorescamine to detect the primary amino groups that have not undergone acylation. The assays were run in dimethylformamide, and, under the conditions employed, Zn-free insulin gave a clear linear response in amounts ranging from 0.5 to 15 nmoles. The fluorescence intensity of a particular sample was expressed relative to the fluorescence intensity of an equimolar amount of Zn-free insulin. The use of insulin as the standard is only arbitrary because it does not take into account possible modifications of the fluorescence yield arising from different degrees of reactivity of buried and exposed amino groups and different sensitivity of individual emitting groups. On the other hand, the values obtained with samples of a given compound are directly comparable. The assay, in addition to being simple and reproducible, proved to be much more sensitive than the previously used ninhydrin method.

The reaction conditions of Boc-protection reaction and the relative fluorescence intensities of the products obtained in typical experiments are summarized in Table II. By comparing experiment no. 1 with no. 2, and no. 3 with no. 4, it follows that the acylating efficiency of the system Boc-azide — 1,1,3,3-tetramethylguanidine — 1,2,4-triazole is higher than that of Boc-azide —

TABLE II

Relative Fluorescence Intensities of di-Boc-Desoctapeptide-insulin Pentamethyl Ester (III), tri-Boc-Insulin (IV) and tri-Boc-Insulin Hexamethyl Ester (V) Prepared Under Various Conditions[a]

Expt. no.	Compound	Conditions of Synthesis		Fluorescence at conc./%:	
		Method[b]	Molar excess of Boc-azide over amino groups	10 nmol/ml	1 nmol/ml
—	Insulin	—	—	100	100
—	(II)[c]	—	—	61.3	60.3
1.	(III)	*A* (4)	315	1.3	n. d.
2.	(III)	*C* (4)	315	8.5	n. d.
3.	(III)	*A* (4)	180	1.2	n. d.
4.	(III)	*B* —	180	4.9	4.7
5.	(III)	*A* (2)	180	1.1	1.2
6.	(III)	*D* —	180	11.1	11.5
7.	(III)	*D* —	315	11.6	11.5
8.	(IV)	*A* (4)	250	1.2	n. d.
9.	(IV)	*A* (2)	120	1.2	1.3
10.	(IV)	*B* —	120	3.5	3.1
11.	(V)	*A* (4)	250	1.1	n. d.

[a] Measured in dimethylformamide at excitation 395 nm and emission 490 nm. The fluorescence intensity is expressed relative to that of Zn-free insulin at the same molar concentration.

[b] Boc-lation performed in the presence of: *A* = 1,1,3,3-tetramethylguanidine + 1,2,4-triazole (mg triazole/μmole substrate), 38 °C, 6 h; *B* = triethylamine, 40 °C, 5 h, ref 2 and 4; *C* = imidazole (mg/μmole substrate), 38 °C, 6 h, ref 7; *D* = 1,1,3,3-tetramethylguanidine, 38 °C, 6 h. All of the products were precipitated with ether, centrifuged off, washed with ether (3 x) and dried.

[c] Desoctapeptide-insulin pentamethyl ester

n. d. = not determined

imidazole and Boc-azide — triethylamine, respectively. As shown in experiments no. 5 and 9, the same acylating efficiency could also be achieved with a reduced amount of triazole; in addition, a variation of experiment no. 9 by shortening the reaction time from 6 to 5 h was without consequences on the relative fluorescence intensity of the product. Experiments no. 6 and 7 revealed that the presence of 1,2,4-triazole is essential for the promotion of the reaction: Boc-lation of II performed in the presence of tetramethylguanidine, but without addition of triazole, led to incomplete substitution, even when larger excesses of Boc-azide were used.

The fully Boc-substituted products prepared by the triazole-promoted reaction were obtained in yields over 90%. For characterization, they were subjected to amino acid analysis and deprotection with trifluoroacetic acid; the amino acid composition of III, prepared in experiment no. 5 is given in Table I. All of the Boc-protected derivatives gave, upon treatment with trifluoroacetic acid, a product which possessed on cellogel electrophoresis (pH 4.8 and 6.5) the same mobilities as desoctapeptide-insulin pentamethyl ester (II), insulin and insulin hexamethyl ester (I), respectively. The product regenerated from IV gave the correct amino acid composition of insulin; it was converted into the Zn-salt which on polyacrylamide electrophoresis (pH 9.5) behaved identically as the starting Zn-insulin and, as determined by the mouse fall test, had a biological activity that was indistinguishable from native insulin.

The data presented suggest that the acylating efficiency of the Boc-azide — 1,1,3,3-tetramethylguanidine — 1,2,4-triazole system might also be usefully applied to semisynthetic work with other peptides and proteins.

EXPERIMENTAL

Materials

Porcine insulin, crystalline zinc-salt, was obtained from PLIVA (lot no. 4,047,022, 24.4 I.U./mg) and was used throughout. Zn-free insulin was prepared[4,15] by adding Na_2EDTA (18.8 mg) to zinc-insulin (500 mg) in 2% acetic acid (25 ml) and adjusting the solution to pH 5.4 with 0.2 mol dm^{-3} NaOH; the resulting precipitate was centrifuged off, washed with water, ethanol and ether and dried. Insulin hexamethyl ester (I) was prepared from Zn-free insulin by the BF_3-MeOH esterification as described by Gattner et al.[9]; the product obtained by precipitation with ether was centrifuged off, washed (3 x) with ether, dried overnight in a desiccator and then used directly in the tryptic digestion step.

Fluorescamin (Fluram) was from Hoffmann La Roche and 1,2,4-triazole (puriss.) from Fluka. *tert*-Butyloxycarbonyl (Boc) azide (prepared from Boc-hydrazide, Fluka) was redistilled before use; fraction with b. p. 64—68 °C/85 Torr* was used. *N,N*-Dimethylformamide (DMF) was distilled over CaH_2 and kept in a stoppered container over molecular sieve (A-4, Fluka). 1,1,3,3-Tetramethylguanidine (Eastman) was redistilled, and fraction with b. p. 91—93 °C/115 Torr* was used. Trypsin, bovine was from Sigma (Type XI, chymotrypsin free).

Methods

All separations on columns were carried out at 4 °C. Products were recovered by evaporation of the eluates to small volume (rotary evaporator, bath temp. < 35 °C) followed by lyophilization.

Electrophoresis on cellulose acetate was performed on strips (2.5 × 14 cm) in a standard electrophoresis apparatus (Chemetron) at 200 V and at pH 4.8 (0.1 mol dm^{-3} pyridine/6 mol dm^{-3} urea buffer adjusted with acetic acid) or at pH 6.5 (0.065 mol dm^{-3} phosphate/7 mol dm^{-3} urea buffer). The strips were stained with 0.25% solution of

* 1 torr = 133.322 Pa

Ponceau S in 3% trichloroacetic acid. Disc-gel electrophoresis was carried out on acrylamide gel, prepared by the standard technique, at pH 9.5 (0.05 mol dm^{-3} Tris/0.03 mol dm^{-3} glycine); cross-linkage of the spacer gel was 2.5% and of running gel 15%. The bands were stained with Coomassie Brilliant Blue R-250 (Sigma).

Amino acid analyses were made on a LKB automatic amino acid analyzer, model BC 200. All hydrolyses were performed in 6 mol dm^{-3} HCl in sealed evacuated tubes at 120 °C for 14 h. Samples were added in 0.2 mol dm^{-3} sodium citrate buffer (pH 2.2), and analyses were run at 55 °C with three sodium citrate buffers: (1) 0.2 mol dm^{-3}, pH 3.25, (2) 0.2 mol dm^{-3}, pH 4.25, and (3) 1.2 mol dm^{-3}, pH 6.45.

The fluorescence intensity produced by the reaction of fluorescamine with primary amino groups was measured on an Farand MK fluorescence spectrophotometer with the excitation wavelength at 395 nm and emission at 490 nm without filter, using $1 \times 1 \times 3$ cm quartz cell. Standard Zn-free insulin and samples (1 μmole each) were prepared as DMF (10 ml) solutions, and aliquots of 0.3 and 0.03 ml, respectively, were taken and made up to 2.5 ml with DMF. Then 0.5 ml of a freshly prepared solution of fluorescamine in acetone (3 mg/10 ml) was added, and the tube was immediately agitated with a vortex mixer. Fluorescence was measured at 25 °C within 10 min and 3 h after addition of the reagent and expressed as relative to that of an equal molar concentration of the standard which had been taken arbitrarily as 100%. A linear response with Zn-free insulin was obtained from 0.5 to 15 nmoles, and no change in fluorescamine-reactive material was seen with time; the contribution of the blank (DMF + fluorescamine) was negligible.

Desoctapeptide-(B^{23-30})-insulin Pentamethyl Ester (II)

The incubation was performed in a thermostated vessel in the pH-Stat (Radiometer Copenhagen, units: pH Meter 26, titrator TTT11 and autoburette ABU 12) under nitrogen atmosphere. The hexamethyl ester I (300 mg, 0.02 mmol) was suspended in water (75 ml), adjusted with 0.1 mol dm^{-3} NaOH to pH 7.5 and, after preincubation at 25 °C for 30 min, trypsin (15 mg) dissolved in 4×10^{-3} mol dm^{-3} hydrochloric acid containing 1×10^{-2} mol dm^{-3} $CaCl_2$ (2.4 ml) was added to the solution. The digestion was allowed to proceed at pH 7.5 (automatic correction of pH with 0.1 mol dm^{-3} NaOH) and 25 °C for 24 h, whereupon the reaction was stopped by adjusting to pH 8.4 with 0.1 mol dm^{-3} NaOH. The resulting precipitate was collected by centrifugation and subjected to fractionation on Sephadex G-50 (fine) as described in Figure 1. After lyophilization of peak tubes, a total of 125.9 mg of fluffy material was obtained which was characterized (Table I) as II free of the starting hexamethyl ester I.

N^{A1}, B^{B1}-*di*-tert-*Butyloxycarbonyl-desoctapeptide-(B^{23-30})-insulin Pentamethyl Ester (III)*

(*a*) *In the presence of 1,1,3,3-tetramethylguanidine and 1,2,4-triazole.* — To a solution of the above II (60 mg, 12 μmole, 24 μequiv. of NH_2 groups) in DMF (6 ml) was added at room temperature 1,2,4-triazole (20 mg, 0.24 mmol) in DMF (1 ml) followed by alternative addition of Boc-azide (614 mg, 178 × excess) and 1,1,3,3-tetramethylguanidine (~ 0.2 mol dm^{-3} in DMF) at such a rate (10 min) that the apparent pH of the reaction mixture was kept (pH meter) within 9.0—9.5. The reaction was allowed to proceed at 38 °C for 6 h, and the pH of the solution was readjusted to 9.4—9.5 with 0.2 mol dm^{-3} tetramethylguanidine solution after the first, third and fifth hour of incubation. After cooling, anhydrous ether was added to the solution, and, after keeping overnight in refrigirator, the resulting precipitate was centrifuged off, washed with ether (3 ×) and dried. The white powder (56.4 mg, 93.9%) was characterized by amino acid composition (Table I), relative fluorescence intensity (Table II, expt. no. 5) and deblocking with trifluoroacetic acid as III.

The same treatment of II (60 mg) but with a higher amount of 1,2,4-triazole (40 mg) and the same or higher amount of Boc-azide (614 and 1.075 mg, resp.) afforded III (yields 92.5—95.3%); the relative fluorescence intensity of these products is given in Table II (expts. no. 1 and 3).

A sample of III was dissolved in DMF-hexamethylphosphoramide (1 : 1, 25 ml) and precipitated by subsequent addition of water (6 ml); after standing overnight in refrigirator, the white crystalline precipitate was centrifuged off, washed with ether (3 ×) and dried; yield: 27 mg. The relative fluorescence intensity of this specimen

was virtually identical (1.2%) to that of III which was not submitted to additional purification.

(*b*) *In the presence of imidazole.* — II (60 mg) was treated with Boc-azide (1 075 mg) and imidazole (40 mg) in DMF (8 ml) exactly as described[7] for the preparation of tri-Boc-insulin to give 55 mg of III which fluorescence intensity relative to insulin is shown in Table II (expt. no. 2).

(*c*) *In the presence of triethylamine.* — The reaction was performed as described by Weitzel et al[4]. to give III (Table II, expt. no. 4) in 91.5% yield.

N^{A1}, N^{B1}, N^{B29} -*tri*-tert-*Butyloxycarbonyl-insulin* (IV)

Samples of insulin hydrochloride[16] (60 mg, 10.2 μmole, 30.6 μequiv. of NH_2 groups) in DMF (6 ml) were treated with Boc-azide (520 mg, 3.63 mmole or 1 075 mg, 7 mmol) in the presence of 1,2,4-triazole (40 or 20 mg) and 1,1,3,3-tetramethylguanidine as described for the preparation of III under (*a*). The ether-precipitated and -washed products were characterized by fluorescence assay (Table II, expts. no. 8 and 9), amino acid composition and deblocking with trifluoroacetic acid; yields: 93.5—95.5%.

N^{A1}, N^{B1}, N^{B29} -*tri*-tert-*Butyloxycarbonyl-insulin Hexamethyl Ester* (V)

Insulin hexamethyl ester (I, 32 mg, 5.5 μmol) in DMF (3 ml) was treated with Boc-azide (580 mg), 1,2,4-triazole (20 mg) and 1,1,3,3-tetramethylguanidine as described for III under (*a*) to give 31.5 mg, 93.2% of V which was characterized by amino acid composition, relative fluorescence intensity (Table II, expt. no. 11) and deprotection with trifluoroacetic acid.

Removal of Boc-Protecting Groups

Deprotection of III, IV and V was performed as described by Levy and Carpenter[2], and the ether-precipitated products were subjected to amino acid analysis and cellogel- and disc-electrophoresis. The precipitated protein obtained by treating IV (20 mg) with trifluoroacetic acid was dissolved in 0.5 mol dm^{-3} acetic acid to which Zn-acetate in 0.5 mol dm^{-3} acetic acid (1 mg/ml, 0.33 ml) was added, and the solution was adjusted with 5 mol dm^{-3} ammonium hydroxide to pH 5.81. After standing overnight in the refrigirator, the precipitate was centrifuged off, washed with ether and dried. On cellogel- and disc-electrophoresis (pH 4.8 and 6.5, and pH 9.5, resp.) the product behaved identically as the starting Zn insulin, and in the mouse fall test it revealed a biological activity equivalent to that of the starting insulin.

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SAŽETAK

Sinteza potpuno Boc-zaštićenih insulinskih derivata s Boc-azid — 1,1,3,3-tetrametilguanidin — 1,2,4-triazolom kao efikasnim acilirajućim sistemom. — Određivanje stupnja *N*-supstitucije produkata s pomoću jednostavne i osjetljive reakcije s fluorescaminom

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Zaštita primarnih amino-skupina s *tert*-butoksikarbonil (Boc) skupinama u desoktapeptid-(B^{23-30})-insulin pentametil esteru, insulinu i insulin-heksametil-esteru provođena je s Boc-azidom u prisutnosti 1,1,3,3-tetrametilguanidina kao baze i 1,2,4-triazola kao aktivatora. Stupanj *N*-supstitucije u izoliranom di-Boc-desoktapeptid-(B^{23-30})-insulin-pentametil-esteru, tri-Boc-insulinu, odnosno tri-Boc-insulin-heksametil-esteru ispitivan je metodom fluorescencije i izražen kao relativni intenzitet fluorescencije prema intenzitetu što ga daje ekvimolarna količina slobodnog insulina. Na taj način ustanovljeno je da je: (*a*) sistem Boc-azid — 1,1,3,3-tetrametilguanidin — — 1,2,4-triazol efikasniji nego do sada opisani sistemi za dobivanje Boc-derivata insulina i njegovih derivata, te da je (*b*) prisutnost 1,2,4-triazola od primarne važnosti za efikasnost acilacije.

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