SYNTHESIS OF [^{3}H-TYR^{B26}]-HUMAN INSULIN BY ENZYMIC SEMISYNTHESIS

Andrè HÄNSICKE[b], Michael BEYERMANN[a], Johannes OEHLKE[a], Erhard MITTAG[c], Uwe KERTSCHER[a], Zhanna D. BESPALOVA[d], Mikhail I. TITOV[d], Michael BIENERT[a]*, Klaus-Dieter KAUFMANN[b] and Hartmut NIEDRICH[a]

[a] *Institute of Drug Research, Academy of Sciences of G.D.R., Alfred-Kowalke-Str. 4, Berlin, G.O.R.-1136*
[b] *VEB Berlin-Chemie, Glienicker Weg 124, Berlin, G.D.R.-1199*
[c] *Central Institute of Nuclear Research, Rossendorf, G.D.R. and*
[d] *USSR Cardiological Research Centre, Akademy of Medical Sciences, 121500 Moscow, 3rd Cherepkovskaya ulitsa, U.S.S.R.*

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Dedicated to the memory fo Dr Karel Bláha.

A procedure is described for tritium labeling of human insulin in position Tyr^{B26} by means of trypsin catalyzed condensation of DiBoc-DOI with [N$^{\varepsilon}$-Boc, ^{3}H-Tyr^{B26}]-IOP**, subsequent deprotection and purification by HPLC. The tritium labeling of the octapeptide was accomplished by dehalotritiation of the corresponding Dit^{B26}-octapeptide which was obtained both by iodination of N$^{\varepsilon}$-Boc-IOP and by total synthesis.

Despite the availability of various powerful methods for tritium labeling of peptides, the synthesis of tritium labeled peptides containing disulfide bridges remains a challenge. The disulfide bridges are known to undergo oxidation when the peptides are iodinated to obtain diiodinated tyrosine and/or histidine peptides as precursors for catalytical dehalotritiation. Additionally, the sulfur atoms disturb the catalytical exchange of halogenes by tritium. Thus, in an initial attempt to obtain tritium labeled insulin by iodination and catalytical dehalotritiation of the entire molecule, complex mixtures were obtained from which ^{3}H-insulin was purified by affinity chromatography[1]. Considerable progress in synthesizing ^{3}H-insulins has been achieved by working out procedures of chemical semisynthesis, allowing the introduction of ^{3}H-Phe into position B1 (ref.[2]) or ^{3}H-Gly into position A1 (ref.[3]), respectively.

* To whom correspondence should be addressed.

** Symbols and abbreviations are used according to the rules of IUPAC IUB Commission on Biochemical Nomenclature, Eur. J. Biochem. *138*, 9 (1984). Dit, L-3,5-diiodotyrosine; DOI, desoctapeptide (B23—30)porcine insulin; IOP, human insulin octapeptide (B23—30).

Using this technique, chemically defined ^{3}H-insulins can be prepared; however, the synthetic route consists in eight different steps. In the last few years, the usefulness of enzymes as catalysts for peptide bond formation in semisynthetic procedures has been demonstrated by different groups. The enzymic semisynthesis offered an elegant access to the preparation of human insulin by coupling Di-Boc-desocta-peptide^{B23-30}-insulin (*I*) and N$^{\alpha}$Boc-insulin octapeptide^{B23-30} (BOC-IOP, *II*) by means of trypsin[4], or trypsin catalyzed transpeptidation of porcine insulin in the presence of a hundred-fold excess of threonine tert. butylester[5,6]. These results prompted us to include procedures of enzymic semisynthesis into our efforts for synthesis of tritiated insulin. In this communication we report on the synthesis of [^{3}H-Tyr^{B26}]-human insulin (*VI*), realized by trypsin-catalyzed coupling of *I* with [N$^{\alpha}$-Boc-^{3}H-Tyr^{B26}]-IOP (*IV*).

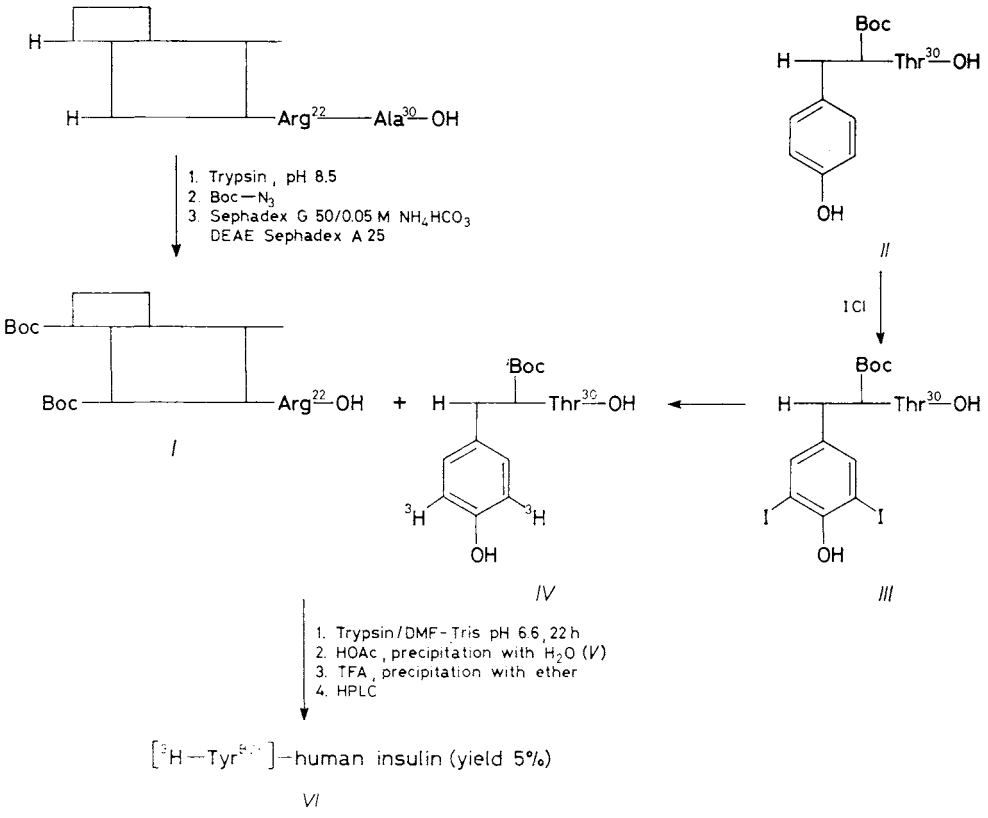

FIG. 1
Scheme for the preparation of [^{3}H-Tyr^{B26}]-human insulin

The synthesis of [^{3}H-Tyr^{B26}]-human insulin was performed as outlined in Fig. 1. The key intermediate *I* was synthesized according to a published procedure[7] by incubation of porcine insulin with trypsin and subsequent purification by gel chromatography on DEAE-Sephadex X-25. The isolated desoctapeptide-insulin was converted into *I* by treatment with tert.butyl azidoformate[8]. It is noteworthy that inhomogeneous and partially insoluble material was obtained when using ditert.-butyl dicarbonate, probably due to acylation of other functional groups (see also[9,10]).

In order to obtain a suitable precursor for the introduction of tritium atoms into the octapeptide *II*, we synthesized the diiodinated peptide *III*, alternatively by direct iodination of *II* and by peptide synthesis using L-diiodotyrosine (Dit). In initial iodination experiments with iodine monochloride in ammonium acetate buffer pH 6·0 (ref.[11]) we were unable to obtain a homogeneous diiodinated peptide, even when the product was subjected to partition chromatography. However, pure samples of *III* were obtained by iodination at pH 2·0 and purification on RP-18 silica gel.

The route for the total synthesis of *III* is outlined in Fig. 2. Dit was coupled without protection of its carboxylic group. The synthesis of Bpoc-Gly-Phe-Phe-Dit-OMe was also accomplished; however, the saponification of the methyl ester failed. Bpoc-

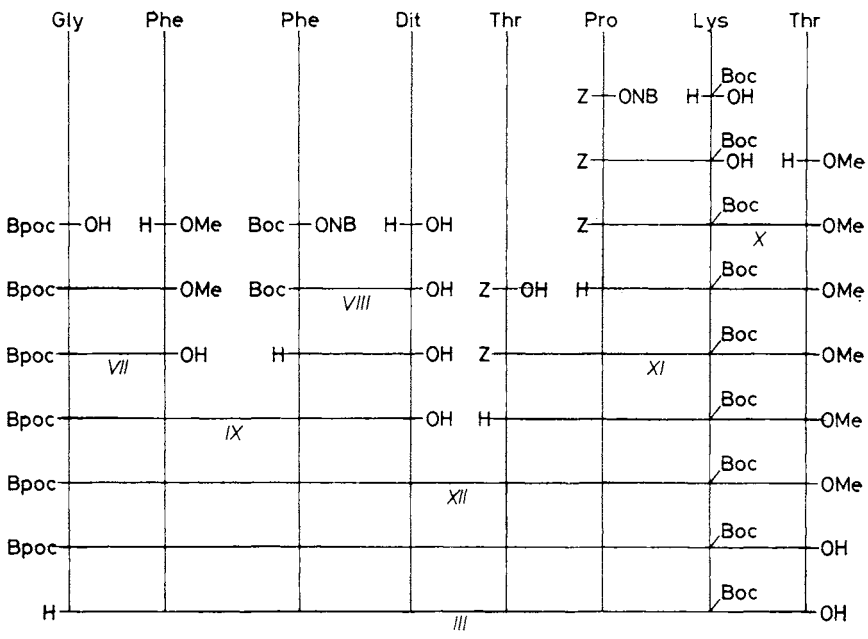

FIG. 2

Synthesis of H-Gly-Phe-Phe-Dit-Thr-Pro-Lys(Boc)-Thr-OH (*III*)

-Gly-Phe-Phe-Dit-OH (*IX*) was purified by gel chromatography and condensed with the C-terminal tetrapeptide by means of DCC/HONB. The octapeptide *XII* was purified by gel chromatography and finally deprotected by saponification and treatment with 80% acetic acid. Both samples of diiodooctapeptide *III* exhibited identical UV-spectra, HPLC and TLC-behavior.

Dehalotritiation of *III* was achieved with tritium gas in the presence of palladium/alumina in aqueous dimethylformamide or dimethylacetamide, yielding *IV* with a specific radioactivity of 0·44 TBq/mmol (12 Ci/mmol) and 0·85 TBq/mmol (23 Ci/mmol), respectively. The improved tritium incorporation when carried out in dimethylacetamide corresponds well to previous results obtained in the deuteration of model compounds[12]. The different batches of *IV*, characterized by TLC and paper electrophoresis, had a sufficient purity and were used for the trypsin catalyzed fragment condensation without further purification.

In published protocols[4] a tenfold excess of Boc-IOP was used to obtain the coupling product with a yield of 55–65%. To achieve an improved utilization of the tritium labeled peptide *IV*, in tracer experiments we studied the coupling reaction with different *I*/*IV*-ratios. A sufficient yield of 40–50% was obtained even with a fivefold excess of *IV* and these conditions were also used successfully in the preparation of highly labeled human insulin. Most of the excess of *IV* (about 80%) remains in the supernatant, when the Tri-Boc-insulin *V* is precipitated by addition of water. After isolation of *V*, the deprotection was performed by treatment with trifluoroacetic acid, and the crude material was purified by HPLC, followed by desalting on a Sephadex G-10 column. HPLC analysis demonstrated that the purified product is free of the starting compounds *I* and *IV* and the corresponding deprotected intermediates DOI and IOP. However, in a few experiments the final product contains small amounts of non-peptidic, UV-active impurities, disturbing the estimation of the insulin concentration in stock solution by UV-measurements, which results in lower values for the specific radioactivity than calculated from RIA-measurements. Using 5 μmol of *IV* with a specific radioactivity of 23 Ci/mmol and 1 μmol of *I*, [^{3}H-Tyr^{B26}]-human insulin *VI* was obtained in a yield of 5%. According to Fig. 1 we prepared different batches of *VI* with specific radioactivities in the range of 0·37 GBq/mmol (10 mCi/mmol)–0·84 TBq/mmol (23 Ci/mmol) (depending on the degree of dilution of *IV* by inactive peptide). The ^{3}H-insulin was stored in 0·5M-acetic acid–ethanol 1 : 1 at −20°C and repurified by HPLC to remove chemical (mainly desamido insulin) and radiochemical decomposition products immediately before use in biopharmaceutical studies (Lorenz et al., manuscript in preparation).

In our efforts to work out a simple method for tritium labeling of insulin we also considered the trypsin-catalyzed exchange of B^{30}-Ala (ref.[5]) by ^{3}H-amino acid esters. However, this approach would require a fifty to hundred-fold excess of the expensive labeled L-amino acid derivative. The use of the better accessible racemic compound was precluded by the low stereospecificity of trypsin. Thus, D-Ala-OMe

is incorporated nearly to the same extent as the L-derivative, when using the standard conditions for the trypsin-catalysed transpeptidation[5] (Beyermann, M. unpublished results). In contrast to the transpeptidation reaction, the trypsin catalyzed coupling of *I* with the labeled octapeptide *IV* requires only a fivefold excess of *IV*. Moreover, the strategy outlined in Fig. 1 should also permit the increase of the specific radioactivity by synthesizing octapeptides containing more than one halogenated and/or unsaturated amino acids.

EXPERIMENTAL

Iodine monochloride solution was prepared according to ref.[13]. N,N-Dimethylacetamide was distilled and stored over molecular sieve. Thin layer chromatography of the octapeptides was carried out on silica gel plates (Kavalier, Czechoslovakia) and silica gel 60 plates (Merck, F.R.G.) in the solvent system pyridine–acetic acid–water–ethyl acetate 20 : 6 : 11 : 45. Solvent fronts were allowed to ascend 10 cm and peptides were visualized using ninhydrin or Fluram reagent. For the detection of radioactive peptide a thin layer scanner from Berthold and Frieseke (F.R.G.) was used. Electrophoresis was performed on paper type FN7 (VEB Papierfabrik Niederschlag, G.D.R.) in 7% acetic acid at 23 V/cm. Polyacrylamide gel electrophoresis was carried out in Tris-HCl buffer pH 8·95 (ref.[16]) For amino acid analysis, 400 nmol samples of the peptides were hydrolyzed in evacuated sealed tubes at 110°C for 20 h and analyzed by amino acid analyzer AAA 881 (Microtechna, Praha, Czechoslovakia).

Tritiations were carried out in a vacuum manifold described elsewhere[14]. Tritium gas was purchased from Techsnab Export (U.S.S.R.) and stored in the form of uranium tritide. 10% Palladium/alumina catalyst was a product of Engelhard (F.R.G.).

HPLC of labeled and non-labeled peptides was carried out on 4·6 × 250 mm LiChrosorb-RP 18 (10 μm) columns, using the solvent system 0·01M phosphate/0·1M-$BaClO_4$ pH 2·2, 60%, and acetonitrile–2–propanol 2 : 1, 40% (isocratic); flow 1 ml/min; detection: 220 and 254 nm. To determine the concentration of *IV* in solution, the reaction with Fluram (La Roche, U.S.A., 15 mg/100 ml acetone) was used accroding to[15]. The concentration dependence of the fluorescence was measured with N^{α}Boc-IOP in 0·5M borate buffer pH 7·8 in the range of 0·5—5 nmol using a Spekol (VEB Carl Zeiss Jena, G.D.R.) spectrophotometer, fitted with the fluorescence equipment FK (365 nm). Ultraviolet absorption spectra were recorded on a Specord M 40 (VEB Carl Zeiss Jena, G.D.R.). Liquid scintillation counting was carried out by a Wallac 81 000 (LKB, Sweden) counter.

Zn-porcine insulin and human insulin were from VEB Berlin-Chemie, G.D.R. DOI was prepared from porcine insulin hydrochloride according to[7] in a yield of 37% and converted into DiBoc-DOI (*I*) by treatment with Boc-azide in dimethylsulfoxide at 37°C (ref.[15]). Both DOI and *I* were found to be homogeneous in HPLC and polyacrylamide gel electrophoresis and exhibited the expected amino acid composition. N^{α}Boc-IOP was prepared by peptide synthesis in solution. Experimental details were published elsewhere[17].

H-Gly-Phe-Phe-Dit-Thr-Pro-Lys(Boc)-Thr-OH (*III*)

Method A: 20 mg *II* (ref.[16]) (19 μmol) were dissolved in 2·5 ml dimethylformamide and diluted with 2·5 ml water. 360 μmol iodine monochloride (800 μl of a 0·4M iodine monochloride solution in 2M-HCl) were added under stirring at 0—4°C and a pH-value of 2 was adjusted by addition of 6M sodium hydroxide solution within 2 min. After a reaction time of 30 min at 0—4°C, the

iodination products were separated on a 10 × 60 mm LiChropep RP 18 (25—40 μm, Merck) column by subsequent elution with 20 ml of water, 20 ml water–methanol 3 : 2, 20 ml methanol, 10 ml methanol–acetic acid 7 : 3 and 20 ml methanol–acetic acid 2 : 3. Evaporation of the methanol–acetic acid fractions gave small amounts of monoiodinated peptide (R_F 0·35), 7 μmol of a mixture of mono- and diiodinated peptide and 5 μmol pure diiodinated octapeptide *III* (R_F 0·40); UV spectrum, $\lambda_{max}(\varepsilon)$: 231 (4 600); m.p. 184—187°C, $[\alpha]_D^{20}$ −23·0° (*c* 1, acetic acid).

Method B: The preparation of *III* was also carried out by solution synthesis using conventional methods of peptide chemistry. The strategy of the synthesis is outlined in Fig. 2 and in Table I analytical data of intermediates are listed. Both samples exhibited identical UV and TLC-behavior.

H-Gly-Phe-Phe-[^{3}H]Tyr-Thr-Pro-Lys(Boc)-Thr-OH (*IV*)

Peptide *III* (6 mg) was dissolved in 1 ml dimethylacetamide, the solution was frozen by liquid nitrogen and after addition of 60 mg 10% palladium/alumina connected to the tritiation manifold[14]. The vessel was evacuated (0·133 Pa) and tritium gas was introduced up to a pressure of 57·2 kPa to allow the saturation of the catalyst. After thawing, catalytic exchange was allowed to proceed for 65 min, with the initial pressure being 89·1 kPa. The catalyst was removed by centrifugation and washed with dimethylacetamide/water. The combined solutions were freeze dried. The lyophilization was repeated twice to remove tritium labeled solvents. The crude *IV*, characterized by paper electrophoresis and TLC was found to be identical with a sample of *II* and was free of mono- and diiodinated precursors. Radioscanning of the TLC-sheeets revealed a radiochemical purity of at least 70%. The specific radioactivity, calculated on the basis of the

TABLE I

Characterization of intermediates of *III*

Peptide	Formula (mol. wt.)	Calculated/found			M.p., °C	$[\alpha]_D^{25}$, *c* 1 (solvent)
		% C	% H	% N		
VII·DCHA	$C_{39}H_{51}N_3O_5.0{\cdot}5\ H_2O$	71·97	8·05	6·46	70—75	+28·5
	(650·9)	71·51	8·06	6·43		(DMF)
VIII	$C_{23}H_{26}I_2N_2O_6.0{\cdot}5\ H_2O$	40·08	3·95	4·06	103—106	−3·3
	(689·3)	39·67	3·97	3·84		(MeOH)
IX	$C_{45}H_{44}I_2N_4O_8.2\ H_2O$	51·05	4·57	5·29	221—226	−6·4
	(1 058·7)	51·03	4·42	5·32		(THF)
X	$C_{29}H_{44}N_4O_9$	58·76	7·48	9·45	117—119	−50·5
	(592·7)	58·62	7·18	8·95		(MeOH)
XI	$C_{33}H_{51}N_5O_{11}.0{\cdot}5\ H_2O$	56·40	7·46	9·96	60—65	−69·9
	(702·8)	56·72	7·33	9·74		(MeOH)
XII	$C_{70}H_{87}I_2N_9O_{16}.3\ H_2O$	51·95	5·79	7·79	121—125	−29·2
	(1 618·4)	52·05	5·67	7·64		(THF)

determination of the peptide content (Fluram method) of a sample, purified by preparative TLC, was calculated to be 0·84 TBq/mmol (23 Ci/mmol).

In a second experiment, 3 mg of *III*, dissolved in 0·2 ml dimethylformamide and 0·3 ml water containing 2 μl triethylamine, were subjected to catalytical dehalotritiation for 60 min at a pressure of 70·2 kPa. After removal of catalyst and exchangable tritium the remaining material (radiochemical purity 80%) was purified by chromatography on a carboxymethyl cellulose column (1·2 × 12 cm, Servacel CM 32, 0·83 meq./g) by elution with water, yielding 0·2 GBq *IV* with a specific radioactivity of 0·44 TBq/mmol (12 Ci/mmol).

[^{3}H-Tyr^{B26}]-human insulin

Peptide *IV* (5 mg, 5 μmol) was dissolved in 30 μl dimethylformamide. To this a solution of *I* (refs[7,8]) (5 mg, 1 μmol) and 0·5 mg trypsin in 30 μl 0·25M-Tris/HCl, pH 8·2 were added. The pH of the resulting reaction mixture was 6·6. The solution was kept for 20 h at 37°C. After addition of 50 μl acetic acid and 250 μl water the formed precipitate was collected by centrifugation and subsequently washed with 250 μl acetone and 250 μl diethyl ether.

Deprotection of the Boc-groups was accomplished by treatment of the dried precipitate with 100 μl trifluoroacetic acid for one hour at 0°C. The deprotected product was precipitated by addition of 1 ml diethyl ether. The precipitate was isolated by centrifugation, and subsequent washing with diethyl ether, yielding 5 mg crude product. Analysis of this product by HPLC revealed a coupling yield of the enzymic coupling reaction of 40%.

The purification of the crude material was realized by HPLC using the conditions described under "General methods". In a typical run, 400 μg crude product, dissolved in 100 μl eluent, were injected and the fractions appearing from 8 to 10 min were collected (retention times: human insulin 9·2 min, DOI 5·5 min, IOP 4·2 min), concentrated by rotatory evaporation under reduced pressure and desalted by chromatography on a Sephadex G-10 (1·2 × 19 cm) column. Elution with 0·5M acetic acid (2 ml fractions) gave in fractions 7 to 9 [^{3}H-Tyr^{B26}]-human insulin, being identical with a human insulin standard in HPLC and polyacrylamide gel electrophoresis. The overall yield was 5% and the specific radioactivity, determined on the basis of RIA-measurements was found to be 0·84 TBq/mmol. The purified product was stored in 0·5M acetic acid–ethanol 1 : 1 (radioactive concentration 10 MBq/mol (0·27 mCi/ml)) at −20°C and was repurified by HPLC before use.

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