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Preparation of Semisynthetic Insulin Analogues from Bis(*tert*-butyloxycarbonyl)-desoctapeptide-insulin Phenylhydrazide: Importance of the Aromatic Region B24–B26†

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ABSTRACT: Semisynthetic analogues of insulin were prepared from derivatives of desoctapeptide-(B23–30)-insulin (DOI). A1,B1-$(Boc)_2$-DOI (di-Boc-DOI) was converted to A1,B1-$(Boc)_2$-DOI-B22-phenylhydrazide (di-Boc-DOI-NHNH-C_6H_5) by the trypsin-catalyzed addition of phenylhydrazine in aqueous organic solvents at pH 6.5 [Canova-Davis, E., & Carpenter, F. H. (1981) *Biochemistry 20*, 7053–7058]. Treatment of di-Boc-DOI-NHNH-C_6H_5 with BNPS-skatole produced the phenyldiimide. The latter was coupled with a variety of protected peptides that, after removal of protecting groups, yielded the following compounds whose biological activities were compared to that of insulin in binding, in stimulation of hexose transport ( ), and in the stimulation of lipogenesis [ ], in terms of percent of insulin activity, all in the isolated epididymal fat cell: di-Boc-DOI 0.2, (0.1), [0.2]; di-Boc-DOI-NHNH-C_6H_5 0.5, (0.2), [0.5]; DOI 0.2, (0.2), [0.1]; DOI-$(Gly)^{B23}$ 0.2, (0.2), [0.1]; DOI-$(Gly\text{-}Phe)^{B23-24}$ 6.3, (6.3), [8.0]; DOI-$(Gly\text{-}Phe\text{-}Phe)^{B23-25}$ 17.0, (25.6), [24.7]; DOI-$(Gly\text{-}Phe\text{-}Phe\text{-}Tyr)^{B23-26}$ 59.0, (50.0), [69.0]. The semisynthetic derivatives represent a stepwise readdition of the aromatic residues near the C terminus of the B chain. A given analogue demonstrated comparable activity in all three biological assays. The results indicate that the stepwise addition of aromatic residues to the B-chain C terminus of DOI produces an increase in insulin-like activity. The biological activity of DOI-$(Gly\text{-}Phe\text{-}Phe\text{-}Tyr)^{B23-26}$, the derivative in which the aromatic region has been completely reassembled, is the same order of magnitude as that of insulin.

The trypsin-catalyzed removal of residues B23–30 of insulin results in the generation of desoctapeptide-insulin (DOI),[1] an insulin analogue possessing strikingly little capacity to elicit insulin-like effects (Young & Carpenter, 1961; Carpenter & Baum, 1962; Kikuchi et al., 1980). The highly aromatic region Phe^{B24}-Phe^{B25}-Tyr^{B26} has been postulated as important for the formation of insulin dimers (Blundell et al., 1972) and as critical for insulin receptor binding (Pullen et al., 1976). Removal of part of this region by the action of pepsin (Gattner, 1975) generates DOI-Gly-Phe-Phe, a derivative with 20% activity.

Several years ago, Ruttenberg (1972) proposed a method for the synthesis of human insulin from porcine insulin. The method suffered from serious deficiencies and has not been replicable (Gattner et al., 1978; Obermeier, 1978). Variations of Ruttenberg's proposal have been used in attempts to produce insulin analogues modified in the B24–26 aromatic region (Shanghai Insulin Research Group, 1973; Weitzel et al., 1976). The products of these attempts were poorly characterized both chemically and biologically, which leads one to question their validity.

Recently, a new method for the preparation of insulin analogues has been developed (Canova-Davis & Carpenter, 1978; Inouye et al., 1979; Canova-Davis & Carpenter, 1980, 1981). In it, trypsin was used to form a peptide bond between the carboxyl of Arg-22 of the B chain of DOI and an amino group, from either a peptide (Inouye et al., 1979) or some other molecule (Canova-Davis & Carpenter, 1978, 1980, 1981). The Japanese group has used their variation of this method, direct peptide coupling, in the preparation of human insulin from porcine DOI and synthetic human octapeptide B23–B30 (Inouye et al., 1979). The direct peptide coupling method was

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[1] Abbreviations: Boc, *tert*-butyloxycarbonyl; BNPS-skatole, 2-[(2-nitrophenyl)sulfenyl]-3-methyl-3′-bromoindolinine; BSA, bovine serum albumin; Bu^t, *tert*-butyl; DEAE, diethylaminoethyl; DOI, desoctapeptide-(B23–30)-insulin; DOI-Gly, DOI-$(Gly)^{B23}$; DOI-Gly-Phe, DOI-$(Gly\text{-}Phe)^{B23-24}$; DOI-Gly-Phe-Phe, DOI-$(Gly\text{-}Phe\text{-}Phe)^{B23-25}$; DOI-Gly-Phe-Phe-Tyr, DOI-$(Gly\text{-}Phe\text{-}Phe\text{-}Tyr)^{B23-26}$; di-Boc-DOI, A1,B1-$(Boc)_2$-DOI; di-Boc-DOI-NHNH-$C_6H_5$, A1,B1-$(Boc)_2$-DOI-B22-phenylhydrazide; DIEA, diisopropylethylamine; DMF, dimethylformamide; Et_3N, triethylamine; Et_2O, diethyl ether; F_3CCOOH, trifluoroacetic acid; HOAc, acetic acid; MeOH, methyl alcohol; Me_2SO, dimethyl sulfoxide; $NHNH_2$, hydrazide; OBu^t, *tert*-butyl ester; ONp, *p*-nitrophenyl ester; Z, benzyloxycarbonyl; THF, tetrahydrofuran; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; tri-Boc-insulin, A1,B1,B29-$(Boc)_3$-insulin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hyroxymethyl)aminomethane; TLC, thin-layer chromatography. Unless otherwise noted all amino acids are of the L configuration.

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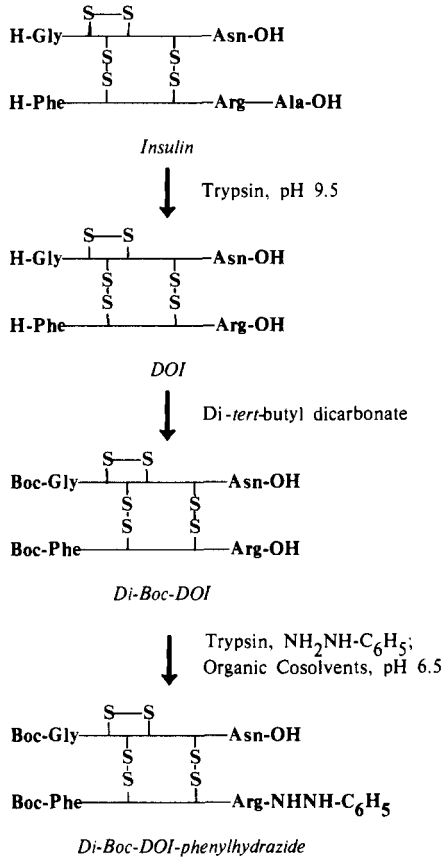
Scheme I: Preparation of Di-Boc-DOI-phenylhydrazide



used in the preparation of a mutant form of human insulin (Tager et al., 1980). Yields in the direct coupling of peptides are variable and appear to be dependent on the structure of the peptide that is being coupled (Gattner et al., 1981).

The method developed in our laboratory (Canova-Davis & Carpenter, 1978, 1980, 1981) uses trypsin-catalyzed coupling to generate a semisynthetic intermediate, di-Boc-DOI-NHNH-C_6H_5. This intermediate can be activated and, in theory, coupled to any primary or secondary amine, regardless of structure. The production of di-Boc-DOI-NHNH-C_6H_5 from di-Boc-DOI along with the activation of di-Boc-DOI-NHNH-C_6H_5 to the phenyldiimide (Milne & Kilday, 1965; Milne & Most, 1968; Milne & Carpenter, 1968) is outlined in Scheme I. The phenyldiimide is sufficiently active to allow the coupling of any peptide to the DOI nucleus. We have used this procedure to produce DOI-Gly, DOI-Gly-Phe, DOI-Gly-Phe-Phe, and DOI-Gly-Phe-Phe-Tyr in a chemically unambiguous manner. The insulin analogues were purified to homogeneity and assayed in three in vitro systems, representing three different steps in glucose homeostasis: insulin binding, hexose transport, and insulin-stimulated lipogenesis (Rieman et al., 1981; Carpenter et al., 1982).

Experimental Procedures

Materials

Zinc was removed from bovine insulin (Eli Lilly and Co., lot 1HJ75J) by gel chromatography on Sephadex G-25 in 10% HOAc (Steiner & Oyer, 1967). Cyanate ion was removed from aqueous urea solutions (Stark et al., 1960) by equilibration with a mixed-bed ion exchanger [Bio-Rad Laboratories, AG-501-X8C(D)] for several hours followed by filtration. Sephadex gel chromatography resins and DEAE-Sephacel were purchased from Pharmacia Fine Chemicals. Reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories. Urea for gel electrophoresis was the ultrapure grade from Schwarz/Mann. Trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK-trypsin) to inhibit contaminant chymotryptic activity (Kostka & Carpenter, 1964; Wang & Carpenter, 1965) was purchased from Worthington Biochemical Corp.

DIEA, 1,4-butanediol, and spectrophotometric-grade Me_2SO were obtained from Aldrich. Dimethylformamide (DMF) was purified by equilibration with 4-Å molecular sieves (Fieser & Fieser, 1969). Tetrahydrofuran (THF) was distilled from $LiAlH_4$ and stored at room temperature under nitrogen. Dry HCl gas and H_2 were purchased from Matheson. Triethylamine (Et_3N, sequanal grade), *N*-ethylmorphine, and BNPS-skatole were from Pierce Chemical Corp. Et_3N was purified before use by distillation from phthalic anhydride (Perrin et al., 1980). Di-*tert*-butyl dicarbonate (99+%), *tert*-butyl nitrite, and Z-L-Phe-ONp were purchased from Fluka AG. L-Phe was from Vega Biochemicals. Z-Gly-ONp, Z-L-Phe-ONp, Gly-OBu^t, L-Phe-OBu^t, and HCl·L-Tyr-(Bu^t)-OBu^t were purchased from Bachem Inc. Other solvents used were reagent grade or better. Palladium on carbon was from Eastman. TLC plates were silica gel 60 plates from Merck, Darmstadt, West Germany.

Crude collagenase and BSA were from Sigma, phloretin was from ICM Pharmaceutical, and Hepes was from Calbiochem. 3-*O*-[^{14}C]Methylglucose, D-[3-^{3}H]glucose, and [^{125}I]iodoinsulin were purchased from New England Nuclear.

Methods

Amino acid analyses were performed on a Beckman-Spinco Model 120B automatic amino acid analyzer (Spackman et al., 1958) equipped with a system AA automatic integrator. Hydrolysis was performed in 6 N HCl containing 0.1% phenol in sealed, evacuated tubes at 110 °C for 20–24 h.

Two systems were employed for polyacrylamide slab gel electrophoresis. (System A) Electrophoresis was performed in the presence of 8 M urea and 0.9 M HOAc at pH 3.5 in 15% acrylamide (Poole et al., 1974). The gel was stained with amido black (Racusen, 1973). (System B) Electrophoresis at pH 8.8 was performed as described by Laemmli (1970) and the gel stained with Coomassie Brilliant Blue R-250. Gel filtration and ion-exchange chromatography separations were monitored with a 2138 Uvicord S detector or by absorbance measurements on a Zeiss spectrophotometer.

Thin-layer chromatography of the synthetic peptides was performed in the following systems: (A) chloroform–MeOH, 98:2; (B) benzene–HOAc, 9:1; (C) 1-butanol–HOAc–water, 4:1:1; (D) chloroform–MeOH, 95:5.

Melting points were recorded on a Thomas-Hoover capillary melting-point apparatus and are uncorrected. $[\alpha]^{24}_{546}$ values were determined on a Bendix Series 100 automatic polarimeter.

Isolation of Adipocytes. Adipocytes were isolated as described by Rodbell (1964) in a pH 7.8 buffer containing 10 mg/mL bovine serum albumin, 0.55 mM glucose, 117 mM NaCl, 6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $NaH_2PO_4 \cdot H_2O$, and 12.5 mM Hepes. Albumin was dialyzed against water for 24 h at 4 °C prior to use in this buffer. Male Wistar rats (150–200 g) were killed by a blow to the head followed by cervical dislocation, whereupon perirenal and epididymal fat pads were removed, washed, and minced. Adipocytes were released by a shaking at 37 °C in a 1 mg/mL solution of crude collagenase. Released cells were filtered through silk screen and rinsed. During the rinse, cells were separated from the buffer containing residual collagenase and cell debris by a 20-s centrifugation at low speed on a clinical

centrifuge. Subsequently, the infranatant solution was removed by aspiration.

In all assays, the bovine insulin standard was tested along with the semisynthetic derivative in question, as a control. Several different concentrations of the standard and the derivative were tested in each assay in order to generate the biological-activity curves.

Lipogenesis Assay. Measurement of the conversion of exogenous glucose to lipids was carried out according to Moody et al. (1974). Adipocytes (10^4) were incubated with shaking at 37 °C for 1–2 h in buffer containing 0.1 μCi of D-[3-^{3}H]glucose and either the bovine insulin standard or an insulin analogue. ^{3}H-Labeled lipids were counted after extraction with toluene-based liquid scintillation fluid.

Hexose Transport Assay. A slight modification of the procedure of Whitesell & Gliemann (1979) was used to assay hexose uptake. Adipocytes (10^5) were preincubated for 10 min in glucose-free buffer containing either the insulin standard or an insulin analogue in a total volume of 110 μL at 37 °C. The reaction was initiated by introducing 10 μL of unlabeled and 0.06 μCi of labeled 3-*O*-[^{14}C]methylglucose such that the final concentration of hexose was 50 μM. This reaction was terminated after 8 s by the introduction of 3 mL of an albumin-free buffer containing 0.3 M phloretin, 0.12% ethanol, and 0.05% Me_2SO. Cells were isolated from the soluble unincorporated radioactive material by centrifugation at 2000*g* for 1 min through a layer of silicone oil. The resultant islets of cells were collected in 2 × 100-μL aliquots with Eppendorf pipets. Radioactivity in the cells and pipet tips was determined by liquid scintillation.

Binding Assay. Inhibition of [^{125}I]iodoinsulin binding was determined by a modification of the method described by Gliemann & Sonne (1978). The 300-μL assay mixture consisted of 10^5 adipocytes, 0.06 μCi of [^{125}I]iodoinsulin, and either the insulin standard or an insulin analogue. The assay mixture was incubated with shaking at 22 °C. After 50 min, the reaction was terminated with the addition of 10 mL of cold 0.9% NaCl. Cells were isolated from the unbound [^{125}I]-iodoinsulin by centrifugation through a layer of silicone oil at 2000*g* for 1 min. The amount of bound radioactivity was determined by γ counting.

Preparation of Desoctapeptide-insulin (DOI). Zn-free insulin (0.5 g, 87.2 μmol) was dissolved in 500 mL of 0.1 M *N*-ethylmorpholine–0.001 M $CaCl_2$ pH 9.5 buffer. A 25-mg aliquot of TPCK-treated trypsin was added, and the mixture was incubated at 4 °C for 24 h. Following lyophilization, the sample was dissolved in 0.1 M NH_4HCO_3, pH 8.0, loaded on a 72 × 3.5 cm Sephadex G-50 fine column and eluted with 0.1 M NH_4HCO_3, pH 8.0. This resulted in the separation of DOI from trypsin, the peptide B23–B29, and alanine. The DOI-containing fractions were pooled and lyophilized.

Preparation of Di-Boc-DOI-phenylhydrazide (Di-Boc-DOI-NHNH-C_6H_5). Both the route and scale of the preparation of di-Boc-DOI-NHNH-C_6H_5 have been revised from the originally published method (Canova-Davis & Carpenter, 1981). In the original procedure, insulin was converted to tri-Boc-insulin. This is treated with trypsin, to give di-Boc-DOI, which was then used for the synthesis of di-Boc-DOI-NHNH-C_6H_5. In contrast, in the current method, insulin is treated with trypsin to give DOI. Di-Boc-DOI is then produced from DOI and di-Boc-DOI-NHNH-C_6H_5 from di-Boc-DOI. The modified procedure is included below.

A sample of DOI (210 mg, 43.6 μmol) was dissolved in 6 mL of Me_2SO containing 150 μmol of Et_3N (15 mg, 21 μL, 1.7 equiv as compared to amino groups). Di-*tert*-butyl dicarbonate (31 μL) was added, and the reaction mixture was stirred at room temperature for 4.5 h. Protein was precipitated from the reaction mixture with a 1:9 (v/v) mixture of MeOH–Et_2O. The product was collected by centrifugation. Following this, the crude di-Boc-DOI was dissolved in 100 mL of 1 M NH_4OH and incubated at 4 °C for 36–40 h. The alkaline treatment was terminated by diluting the sample with 300 mL of water and lyophilizing.

Crude di-Boc-DOI (160 mg, 31.9 μmol) was dissolved in 28 mL of dry Me_2SO, followed by the addition of 28 mL of 1,4-butanediol. The protein solution was cooled in an ice–water bath. To this solution was added 20 mL of 1.83 M phenylhydrazine in water (4.5 mL of phenylhydrazine made to 25 mL with water, 1500 equiv, pH adjusted to 6.5 with 10% HOAc) dropwise over 3 h, followed by 4 mL of ice-cold 5 mM $CaCl_2$. To initiate di-Boc-DOI-NHNH-C_6H_5 synthesis, 4 mg of TPCK-trypsin was added and the reaction mixture was incubated in the absence of light at room temperature. Over a period of 52 h, five more 4-mg aliquots of TPCK-trypsin were added, at 4–10-h intervals. The reaction was terminated by dialyzing the mixture against 8 L of 10% HOAc at 4 °C for 48 h in the dark. The dialysis solvent was changed 5 times during this period. Following dialysis, the crude product was lyophilized.

The di-Boc-DOI-NHNH-C_6H_5 was purified on DEAE-Sephacel ion-exchange resin (2.5 × 45 cm) at 4 °C equilibrated in 0.065 M NaCl, 0.01 M Tris, pH 7.2, and 7 M urea. Following collection of the appropriate peak, as determined by gel electrophoresis in systems A and B, the product was dialyzed against water for 48 h, at 4 °C, with five solvent changes. The yield was 62 mg of di-Boc-DOI-NHNH-C_6H_5 (12.1 μmol, 38% on the basis of crude di-Boc-DOI).

Synthesis of Peptides. All protected dipeptides were synthesized by the nitrophenyl ester coupling method (Bodanszky, 1979; Bodanszky et al., 1976). The protected dipeptides (with one exception) were purified by crystallization. Z-Gly-Phe-OMe could only be isolated as an oil. Since it was to be used in a fragment-coupling synthesis, it was converted to the hydrazide (Meienhoefer, 1979), which was purified by crystallization. Analytical data for the purified protected dipeptides are included in Table I.

Z-Gly-Phe-Phe-OBu^t. Z-Phe-Phe-OBu^t was converted to HOAc·Phe-Phe-OBu^t (11 mmol) by Pd on carbon (10%) catalyzed hydrogenolysis in MeOH containing HOAc. The HOAc·Phe-Phe-OBu^t was added to CH_2Cl_2 and neutralized with DIEA (1.5 equiv). To this solution was added 11 mmol of Z-Gly-ONp, and the reaction was allowed to proceed for 3 days. The product was washed with 5% $NaHCO_3$ and 1 M citric acid. Z-Gly-Phe-Phe-OBu^t was crystallized and recrystallized from EtOAc–hexane. Analytical data for the tripeptide are included in Table I.

Synthesis of Z-Gly-Phe-Phe-Tyr(Bu^t)-OBu^t. A fragment strategy was employed for the synthesis of the tetrapeptide. HCl·Phe-Tyr(Bu^t)-OBu^t was prepared from Z-Phe-Tyr-(Bu^t)-OBu^t by hydrogenolysis over Pd on carbon (10%) in MeOH containing 1 equiv of HCl. Z-Gly-Phe-$NHNH_2$ was converted to Z-Gly-Phe-azide by treatment with *tert*-butyl nitrite and HCl–THF in DMF at −20 °C (Romovacek et al., 1979). Following activation to the azide, the reaction mixture was cooled to −30 °C, and the pH was raised to 8–8.5 (moist pH paper) with Et_3N. A solution of HCl·Phe-Tyr(Bu^t)-OBu^t and Et_3N in DMF was added to the azide solution at −10 °C. The reaction mixture was stirred at 4 °C for 40 h, with Et_3N added at intervals to maintain the pH at 8–8.5. The crude product was washed with 5% $NaHCO_3$ and 1 M citric acid.

Table I: Analytical Properties of Synthetic Peptides

structure	TLC[a] system 1 (R_f)	TLC[a] system 2 (R_f)	[α][b]	mp (°C)	C, H, N calcd (obsd)
Z-Gly-Phe-OBu^t	A (0.60)	B (0.47)	+7.4	73–76	66.97, 6.84, 6.79 (66.98, 6.85, 6.84)
Z-Phe-Phe-OBu^t	A (0.76)	B (0.55)	−20.0	94–96	71.69, 6.82, 5.57 (71.62, 6.80, 5.51)
Z-Gly-Phe-Phe-OBu^t	A (0.37)	B (0.39)	−9.0	118–122	68.68, 6.66, 7.51 (68.76, 6.66, 7.60)
Z-Gly-Phe-$NHNH_2$	A (0.40)	C (0.71)	+0.58	143–145	61.61, 5.99, 15.13 (62.04, 5.99, 15.09)
Z-Phe-Tyr(Bu^t)-OBu^t	A (0.59)	B (0.51)	−13.0	102–105	71.06, 7.37, 4.87 (70.99, 7.36, 4.77)
Z-Gly-Phe-Phe-Tyr(Bu^t)-OBu^t	D (0.56)	B (0.25)	−19.8	112–115	69.39, 6.99, 7.19 (69.44, 7.04, 7.05)

[a] TLC systems: (A) chloroform–MeOH, 98:2; (B) benzene–HOAc, 9:1; (C) 1-butanol–HOAc–H_2O, 4:1:1; (D) chloroform–MeOH, 95:5. Thin-layer chromatography run on silica gel 60 plates, Merck, Darmstadt, West Germany. [b] Optical rotations done on a Bendex Series 100 automatic polarimeter, at 546 nm, T = 24 °C. All rotations measured on solutions (1%) of the various peptides in MeOH except Z-Phe-Phe-OBu^t (MeOH, 0.5%) and Z-Gly-Phe-$NHNH_2$ (DMF, 10%).

The protected tetrapeptide was crystallized from 2-butanone–hexane. Analytical data are included in Table I.

Preparation of Semisynthetic Insulin Derivatives. Prior to activation to the phenyldiimide, di-Boc-DOI-NHNH-C_6H_5 was gel filtered on a Sephadex G-25 fine column (2.5 × 45 cm) in 10% HOAc at 4 °C, to remove any residual salts. The sample size for these desalting chromatograms was 60–80 mg. The product was lyophilized, giving a product with only moderate solubility in aqueous buffers. Di-Boc-DOI-NHNH-C_6H_5 is unstable in this salt-free form, and so this procedure was performed immediately before use.

Peptides that were to be coupled to di-Boc-DOI-NHNH-C_6H_5 were first hydrogenated over Pd on carbon (10%) in the presence of HCl to remove amino-terminal Z protection. The resulting peptides were characterized by thin-layer chromatography and in some instances by elemental analysis. As an example, the coupling of Gly-Phe-Phe-Tyr(Bu^t)-OBu^t to di-Boc-DOI-NHNH-C_6H_5 is detailed below.

Di-Boc-DOI-NHNH-C_6H_5 (61.5 mg, 12.0 μmol) was dissolved in 2 mL of amine-free DMF containing 1.1 equiv of pyridine (13 μmol). A 100-fold excess of phenol was added to the reaction mixture as a scavenger, followed by a 50-fold excess of BNPS-skatole. The BNPS-skatole serves as a mild oxidizer, converting the phenylhydrazide to the phenyldiimide. This reaction was allowed to proceed for 30 min at room temperature in the dark.

After this period, 100 equiv (1.20 mmol) of HCl·Gly-Phe-Phe-Tyr(Bu^t)-OBu^t was added to the reaction mixture in 3 mL of amine-free DMF. This was followed by the addition of 150 equiv (1.80 mmol) of Et_3N. The reaction mixture was stirred in the dark at room temperature for 24 h.

The product was precipitated by the addition of nine volumes of ice-cold Et_2O and collected by centrifugation. The pellet was redissolved in MeOH, reprecipitated by addition of nine volumes of ice-cold Et_2O, and collected by centrifugation. The pellet was dissolved in 3 mL of HOAc, and 8 mL of water was added. The turbid solution was centrifuged, and the supernatant solution was applied to a Sephadex G-50 (fine) column (2.5 × 88 cm) eluted with 20% HOAc. Immediately following its appearance, the product peak (crude yield, 2.3 μmol, 20% on the basis of di-Boc-DOI-NHNH-C_6H_5) was pooled and lyophilized, to minimize the time spent in acidic media. The product was incubated for 24 h in pH 8.0, 0.1 M NH_4HCO_3, followed by lyophilization. The crude product was purified by ion-exchange chromatography on a DEAE-Sephacel column (2.5 × 47 cm) at 4 °C (yield 1.3 μmol, 11% on the basis of di-Boc-DOI-NHNH-C_6H_5). The buffer used for chromatography was 0.01 M Tris, pH 7.2, and 7 M urea, with a 0.065–0.15 M NaCl gradient. The material in the various peaks was pooled, dialyzed against water at 4 °C, and lyophilized. The product (as identified by gel electrophoresis in systems A and B) was stirred in 2 mL of F_3CCOOH–anisole (9:1) for 2 h at room temperature. The sample was diluted to 10 mL with 10% HOAc, extracted once with 10 mL of Et_2O to remove as much anisole as possible, and chromatographed on a Sephadex G-25 column (2 × 34 cm) in 10% HOAc. The product peak was collected and lyophilized. The yield of DOI-Gly-Phe-Phe-Tyr was 3.2 mg or 5% on the basis of the amount of di-Boc-DOI-NHNH-C_6H_5 used as starting material.

Results

Synthetic Peptides. Analytical data for the synthetic peptides are included in Table I. The synthetic peptides all met standard criteria for purity.

Coupling of Synthetic Peptides. Coupling yield is a measure of the efficiency of formation of the peptide bond in a coupling reaction involving a carboxyl component and an amino component. For the purpose of this paper, the carboxyl component is di-Boc-DOI-phenyldiimide, generated from di-Boc-DOI-NHNH-C_6H_5, and the amino component is the particular synthetic peptide used in the synthesis of the desired analogue.

The yields of coupling of the various synthetic peptides to di-Boc-DOI-phenyldiimide were assessed by gel electrophoresis (system B) following 0.1 M NH_4HCO_3, pH 8 treatment of the crude semisynthetic derivatives. In model experiments with di-Boc-DOI-NHNH-C_6H_5, 24-h, room-temperature treatment in this media completely decomposed di-Boc-DOI-NHNH-C_6H_5 to di-Boc-DOI (data not shown). For this reason, it is not advisable to chromatograph or lyophilize di-Boc-DOI-NHNH-C_6H_5 from NH_4HCO_3 buffers. It is reasonable to expect this same decomposition to occur in other alkaline aqueous buffers. This treatment converted di-Boc-DOI-NHNH-C_6H_5 and di-Boc-DOI-phenyldiimide to di-Boc-DOI, while leaving coupled product with a protected B-chain carboxy terminus. Thus, coupled product would have one less negative charge than the starting material, di-Boc-DOI-NHNH-C_6H_5 that had been converted to di-Boc-DOI. This charge difference allowed assessment of coupling yields by gel electrophoresis, along with purification of the semisynthetic product by DEAE-Sephacel chromatography. Coupling yields were estimated by visual inspection of electrophoretic gels run in system B. Coupling yields could be best assessed at this point, since it was the coupling yield and not the overall recovered yield that was of interest at this step. Overall yields were significantly less than the observed coupling yields due to losses during the purification because much of the material was irreversibly bound to the resins. Overall yields are given below.

Coupling yields ranged from near 100% for Gly-OBu^t to approximately 80% for Gly-Phe-Phe-Tyr(Bu^t)-OBu^t. These represent a marked improvement over previously reported

Table II: Amino Acid Analysis Ratios for Semisynthetic Derivatives[a]

amino acid	DOI		DOI-Gly		DOI-Gly-Phe		DOI-Gly-Phe-Phe		DOI-Gly-Phe-Phe-Tyr		insulin	
	calcd	found	calcd	found	calcd	found	calcd	found	calcd	found	calcd	found
Asx	3	3.04	3	3.00	3	2.97	3	3.08	3	2.88	3	2.89
Thr	0	<0.05	0	<0.05	0	<0.05	0	<0.05	0	<0.05	1	0.98
Ser	3	2.75	3	2.35	3	2.81	3	2.45	3	2.52	3	2.58
Glx	7	6.95	7	7.00	7	7.04	7	6.93	7	7.12	7	7.11
Pro	0	abs	0	abs	0	abs	0	abs	0	abs	1	pres
Gly	3	3.09	4	3.96	4	4.05	4	3.92	4	4.02	4	4.08
Ala	2	2.17	2	1.94	2	2.03	2	1.94	2	1.98	3	3.11
$^1/_2$-Cys	6	4.89	6	4.75	6	4.77	6	4.56	6	4.72	6	5.00
Val	5	4.28	5	4.50	5	4.08	5	4.56	5	3.89	5	4.01
Ile	1	0.44	1	0.53	1	0.39	1	0.64	1	0.45	1	0.47
Leu	6	6.11	6	5.80	6	5.67	6	5.95	6	5.81	6	5.92
Tyr	3	3.00	3	2.76	3	2.83	3	2.60	4	4.03	4	4.02
Phe	1	0.95	1	0.96	2	1.90	3	2.81	3	2.94	3	3.00
Lys	0	<0.05	0	<0.05	0	<0.05	0	<0.05	0	<0.05	1	1.03
His	2	1.92	2	2.02	2	1.92	2	2.03	2	2.15	2	2.10
Arg	1	0.99	1	0.96	1	0.96	1	0.94	1	0.91	1	0.93

[a] Values are the average of two hydrolyzates. No correction for destruction of sensitive residues during hydrolysis. Proline values were not integrated. Amino acid residue values normalized to Asx + Glx = 10 residues. Abbreviations: DOI, desoctapeptide-(B23–30)-insulin; DOI-Gly, DOI-(Gly)^{B23}; DOI-Gly-Phe, DOI-(Gly-Phe)$^{B23-24}$; DOI-Gly-Phe-Phe, DOI-(Gly-Phe-Phe)$^{B23-25}$; DOI-Gly-Phe-Phe-Tyr, DOI-(Gly-Phe-Phe-Tyr)$^{B23-26}$; insulin, bovine insulin; abs, absent; pres, present.

coupling yields for nonnatural sequences by direct trypsin activation (Gattner et al., 1981).

The original strategy for the synthesis of DOI-Gly-Phe-Phe-Tyr involved the coupling of Gly-Phe-Phe-Tyr-OBu^t (tyrosine phenolic hydroxyl unprotected) to di-Boc-DOI-NHNH-C_6H_5. A coupling yield of approximately 50% was observed with this tetrapeptide derivative in marked contrast to the 80% coupling yield observed for the globally protected Gly-Phe-Phe-Tyr(Bu^t)-OBu^t. This was probably due to the O-acylation of tyrosine in the synthetic peptide by the carboxyl of Arg-B22. This byproduct decomposed in the NH_4HCO_3 treatment yielding di-Boc-DOI and resulted in the lower coupling yield. Global protection for the synthetic peptide resulted in an improved coupling yield.

Purification of Di-Boc-DOI-Gly-Phe-Phe-Tyr(Bu^t)-OBu^t. Figure 1 represents the DEAE-Sephacel profile for the chromatographic purification of di-Boc-DOI-Gly-Phe-Phe-Tyr(Bu^t)-OBu^t, along with the electrophoretic behavior in system B of the various peaks. Several peaks are apparent. Peak I represents a mono-Boc-DOI-Gly-Phe-Phe-Tyr(Bu^t)-OBu^t derivative. Peak II represents purified di-Boc-DOI-Gly-Phe-Phe-Tyr(Bu^t)-OBu^t. Peak III was not further characterized, peak IV represents di-Boc-DOI, and peak V represents polymer. The gel reveals that the desired product, di-Boc-DOI-Gly-Phe-Phe-Tyr(Bu^t)-OBu^t, is free of di-Boc-DOI. A rather conservative pooling was done at this stage, to ensure purity of the product.

Amino Acid Ratios, Gel Electrophoresis, and Yields for the Final Semisynthetic Analogues. Following complete deprotection in F_3CCOOH and gel filtration of the analogues, amino acid ratios were determined. These analyses are included in Table II. The ratios for insulin and DOI are also included. The data show that the semisynthetic derivatives have the requisite ratios.

In addition, gel electrophoreses were run on the completely deprotected analogues in systems A and B. These gels indicated homogeneity of the products.

Yields of the pure insulin analogues, on the basis of the amount of di-Boc-DOI-NHNH-C_6H_5 used for the synthesis of the analogues, were as follows: DOI-Gly, 2.9 mg, 13%; DOI-Gly-Phe, 7.8 mg, 20%; DOI-Gly-Phe-Phe, 2.3 mg, 7%; DOI-Gly-Phe-Phe-Tyr, 3.2 mg, 5%.

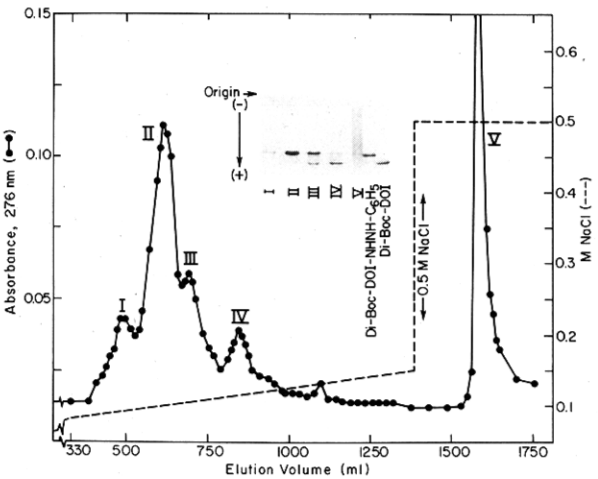


FIGURE 1: Chromatography of di-Boc-DOI-Gly-Phe-Phe-Tyr-(Bu^t)-OBu^t on DEAE-Sephacel (2.5 × 47 cm) with 0.01 M Tris (pH 7.2) and a 0.065 (700 mL)–0.15 M (700 mL) NaCl gradient (– –) in 7 M urea at 4 °C. (Inset) Gel electrophoresis at pH 8.8 (system B) of the various fractions eluted from the column.

Biological Activity of Semisynthetic Intermediates. The biological activities of the semisynthetic intermediates, along with tri-Boc-insulin, in the three in vitro assay systems are shown in Figure 2. The percent activities as compared to that of insulin for these derivatives are shown in Table III.

Biological Activity of Semisynthetic Insulin Analogues. The biological activities of the semisynthetic derivatives in the in vitro assay systems are shown in Figure 3. The percent activities as compared to insulin for these derivatives are shown in Table III.

Discussion

The synthesis of peptide bonds through the reverse action of proteolytic enzymes has received considerable attention in the past few years (Laskowski, 1978). Inouye's group (Inouye et al., 1979) has used trypsin for the direct coupling of human insulin octapeptide B23–B30 to porcine DOI, producing human insulin. This method has also been used to produce semisynthetic mutant human insulin (Tager et al., 1979) by both the Japanese group (Inouye et al., 1981a) and others (Tager et al., 1980; Gattner et al., 1980) and to prepare other sem-

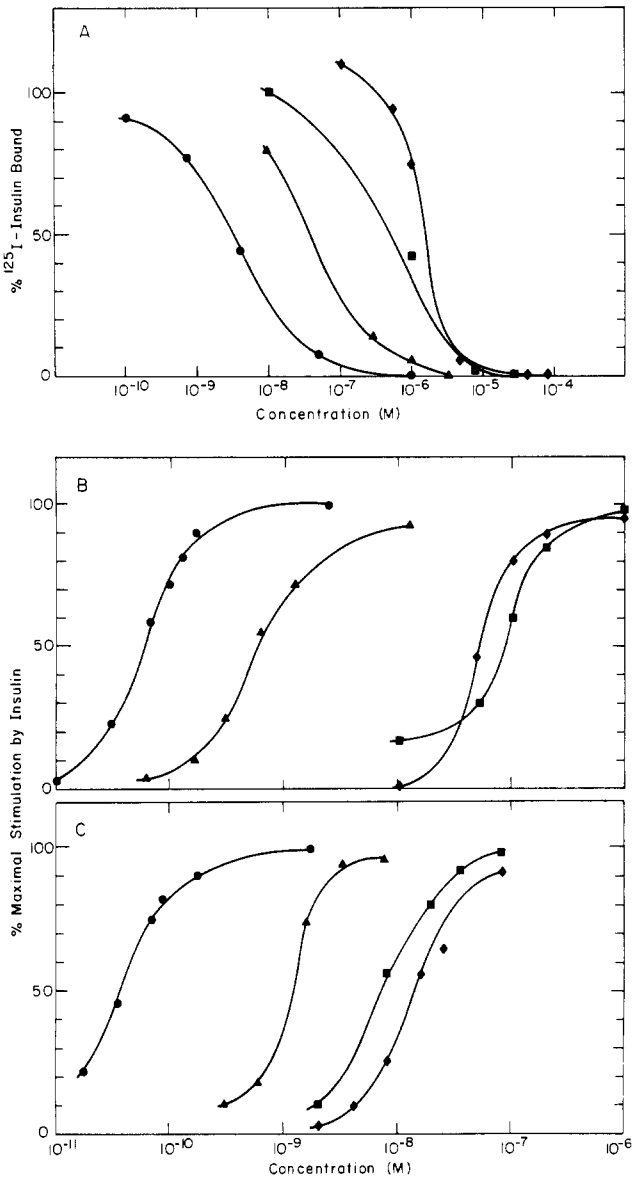


FIGURE 2: Dose–response of semisynthetic insulin intermediates in three assay systems with rat epididymal fat cells: bovine insulin standard (●); tri-Boc-insulin (▲); di-Boc-desoctapeptide-insulin (♦); di-Boc-desoctapeptide-insulin phenylhydrazide (■). (Panel A) Displacement of [^{125}I]iodoinsulin, binding assay; (panel B) hexose transport; (panel C) stimulation of lipogenesis.

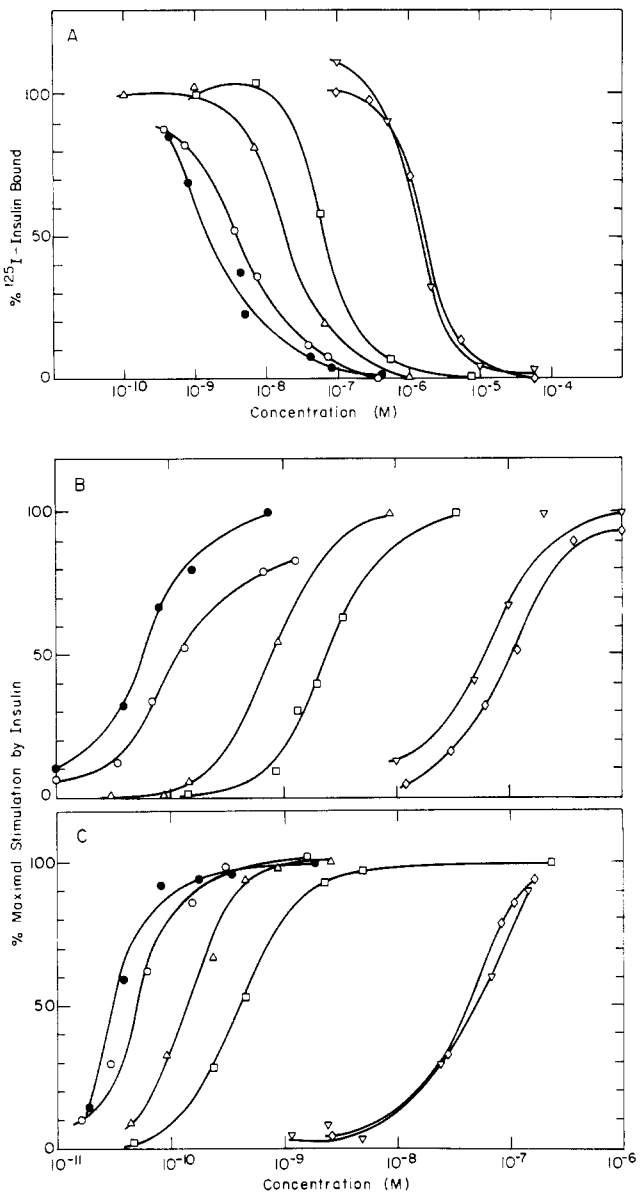


FIGURE 3: Dose–response of semisynthetic insulin analogues in three assay systems with rat epididymal fat cells: bovine insulin standard (●); desoctapeptide-insulin (DOI) (▽); DOI-Gly (◇); DOI-Gly-Phe (□); DOI-Gly-Phe-Phe (△); DOI-Gly-Phe-Phe-Tyr (○). (Panel A) Displacement of [^{125}I]iodoinsulin, binding assay; (panel B) hexose transport; (panel C) stimulation of lipogenesis.

isynthetic insulin derivatives (Inouye et al., 1981b; Chu et al., 1981; Gattner et al., 1981).

We have used the peptide bond forming capacity of trypsin with a different aim. The goal was to prepare derivatives of DOI in which Arg-B22 is specifically activated. The scheme envisaged the use of trypsin to add a component to Arg-B22, which, in a later stage, could be activated chemically to give a reactive carboxyl group. The selective addition of a component to Arg-B22 would make use of the specificity of trypsin. Several components were investigated, among which hydrazine and phenylhydrazine were the most successful (Canova-Davis & Carpenter, 1978, 1980, 1981). Under appropriate conditions, trypsin catalyzes the addition of phenylhydrazine to di-Boc-DOI to form di-Boc-DOI-phenylhydrazide in good yield (60–80%, Canova-Davis & Carpenter, 1981). Mild oxidation of the latter compound yields di-Boc-DOI-phenyldiimide, which contains a highly activated carboxyl component similar to those generally used in peptide synthesis (Rich & Singh, 1979). This results in superior coupling yields as compared to direct trypsin mediated coupling (Gattner et al., 1981) of sequences differing from normal octapeptide B23–B30.

The final isolated yields of the analogues were low. However, they were in the same range as those reported by Gattner et al., (1980) for the preparation of derivatives with substitutions made in the aromatic region B24–B26 of the coupled octapeptide. The yields reported here were slightly lower than those reported by the Japanese group (Inouye et al., 1981b) for analogues with substitutions made in the octapeptide B23–B30. Both these groups prepared derivatives that contained Lys-B29 and thus could be purified from the starting materials by ion-exchange chromatography of the deprotected products. The derivatives prepared in this report all lacked Lys-B29 and had to be purified in the protected form. This contributed to the lower yields in the isolated products.

In an attempt to produce DOI-Gly-Phe-Phe-Tyr with minimal protection of the synthetic peptide (Gly-Phe-Phe-Tyr-OBu^t), a significant drop in coupling yield was observed. We attribute this drop to O-acylation of the Tyr residue in

Table III: Biological Potency[a] of Semisynthetic Intermediates and Insulin Analogues

compound	% of insulin activity		
	binding	activity transport	lipogenesis
insulin (bovine)	100.0	100.0	100.0
tri-Boc-insulin	5.0	5.7	8.0
di-Boc-DOI	0.2	0.1	0.2
di-Boc-DOI-NHNH-C_6H_5	0.5	0.2	0.5[b]
DOI	0.2	0.2	0.1
DOI-Gly	0.2	0.2	0.1
DOI-Gly-Phe	6.3	6.3	8.0
DOI-Gly-Phe-Phe	17.0	25.6	24.7
DOI-Gly-Phe-Phe-Tyr	59.0	50.0	69.0

[a] Biological potency (% of insulin activity) is determined as follows: $(C_i/C_d) \times 100$ = biological potency, where C_i = concentration of insulin required to stimulate half-maximal response and C_d = concentration of derivative required to stimulate half-maximal response. Abbreviations: DOI, desoctapeptide-(B23–30)-insulin; tri-Boc-insulin, A1,B1,B29-(*tert*-butyloxycarbonyl)$_3$-insulin; di-Boc-DOI, A1,B1-(Boc)$_2$-DOI; di-Boc-DOI-NHNH-C_6H_5, A1,B1-(Boc)$_2$-DOI-B22-phenylhydrazide; DOI-Gly, DOI-(Gly)^{B23}; DOI-Gly-Phe, DOI-(Gly-Phe)$^{B23-24}$; DOI-Gly-Phe-Phe, DOI-(Gly-Phe-Phe)$^{B23-25}$; DOI-Gly-Phe-Phe-Tyr, DOI-(Gly-Phe-Phe-Tyr)$^{B23-26}$. [b] A biological potency of 0.01% in the lipogenesis bioassay was found for a sample of di-Boc-DOI-NHNH-C_6H_5 prepared by a different procedure (Canova-Davis & Carpenter, 1981).

the synthetic peptide. This side reaction of Tyr has been frequently observed when an excess of amine is used to neutralize the amino component in a coupling reaction (Ramachandran & Li, 1963; Inman, 1981; Stewart, 1981). For this reason, it is recommended that either global protection of the synthetic peptide or a carefully controlled amount of neutralizing amine be used when phenyldiimide coupling is attempted.

The three semisynthetic intermediates DOI, di-Boc-DOI, and di-Boc-DOI-NHNH-C_6H_5, along with DOI-Gly, have similar low biological potencies in the three assay systems. These compounds stimulate full biological activity when tested in sufficiently high quantities. Recently, it has been shown (Kikuchi et al., 1980) that highly purified DOI has an intrinsic biological activity that is close to the activities we have observed for the above derivatives. This leads us to believe that the <1% activities of DOI, di-Boc-DOI, di-Boc-DOI-NHNH-C_6H_5, and DOI-Gly are native to these compounds and not due to insulin contamination. The low activity of the semisynthetic intermediates and DOI-Gly indicates that once the aromatic region B24–B26 is removed from a derivative, other manipulations, short of reintroducing this region, will not enhance biological activity.

In contrast to the DOI-based derivatives, tri-Boc-insulin, an intermediate from our earlier semisynthetic protocol (Canova-Davis & Carpenter, 1981), contains the aromatic region B24–B26 intact. An earlier preparation of tri-Boc-insulin demonstrated 30% biological activity in the in vivo mouse convulsion assay (Levy & Carpenter, 1967). This is significantly higher than ~6% activity observed in the three in vitro assays (Table III) and is probably due to loss of Boc-groups during the convulsion assay. In any case, the observed activity of tri-Boc-insulin is diminished, despite the presence of the B24–B26 aromatic region.

The addition of a Boc group to the A1 α-amino group causes perturbations in the crystal structure of insulin (Pullen et al., 1976). Along with the displacement of the A2–A5 segment, the side chain of Tyr-A19 is shifted, and residues B24–B28 are slightly displaced. Since modification of the B1 α-amino and B29 ϵ-amino groups seems to have only small effects on biological activity (Blundell et al., 1972), the structural perturbations caused by the A1 Boc group must account for the lower biological potency of tri-Boc-insulin.

Katsoyannis has used another method to prepare some of the derivatives we have prepared with di-Boc-DOI-NHNH-C_6H_5. His strategy entails the combination of a completely synthetic B chain with porcine A chain (Katsoyannis et al., 1973, 1974). Preparation of the synthetic B chain is extremely tedious, and the procedure is further complicated by the need to form the correct disulfide bonds upon recombination of the two chains. Chemical characterization of these semisynthetic derivatives was insufficient, considering the complex strategy used in their preparation. Biological characterization was limited to radioimmunoassay and the mouse convulsion assay. Radioimmunoassay results are ambiguous since the antibodies may react with other parts of the molecule irrespective of the presence of properly paired disulfides. The mouse convulsion assay suffers from the fact that the compounds may undergo metabolic modifications before reaching the target cells.

In contrast, the disulfide bonds of insulin are maintained in di-Boc-DOI-NHNH-C_6H_5. Further, the use of di-Boc-DOI-NHNH-C_6H_5 allows for minimal chemical synthesis in the production of truncated B-chain insulin derivatives. The insulin derivatives in this paper were biologically characterized in three different in vitro assay systems that could be well controlled. The results are certainly more relevant than radioimmunoassay and not subject to the above-mentioned criticism of the convulsion assay.

Insulin analogues similar to those prepared by the use of di-Boc-DOI-NHNH-C_6H_5 have reportedly been prepared from the pentamethyl ester of di-Boc-DOI (Shanghai Insulin Research Group, 1973; Weitzel et al., 1976). Serious deficiencies exist with this method (Gattner et al., 1978; Obermeier, 1978), and results obtained using it are at best ambiguous. Chemical characterizations of the semisynthetic products by this method are meager. Biological characterizations were limited to a single type of whole animal assay in both cases, and in one (Shanghai Insulin Research Group, 1973), an extremely small number of animals were used. It is difficult to draw conclusions from this work.

The biological activity we report for DOI-Gly-Phe-Phe correlates well with that reported for this derivative as prepared by limited peptic digestion and assayed in an in vitro system (Gattner, 1975). The derivatives we have prepared allow for a step by step comparison of the addition of the aromatic region in three different assays representative of three aspects of insulin metabolism.

The stepwise readdition of the aromatic residues results in an increase in activity. This increase is equally reflected in the binding, hexose transport, and lipogenesis assays. With the addition of Phe-B24, the first aromatic residue, the most dramatic increase in activity is seen, approximately 70-fold to 7%. The second aromatic, Phe-B25, moderately increases the activity, and the addition of Tyr-B26 brings the activity to between 50–69% (Table III). DOI-Gly-Phe-Phe-Tyr thus has biological activity of the same order of magnitude as insulin, whereas the starting material, DOI, has biological activity 3 orders of magnitude less than insulin. The three derivatives will stimulate full biological response if given in sufficient quantity.

Since binding is the first step in the physiological action of insulin and since the hexose transport and lipogenesis activities reflect the binding potency, the enhancement that is seen upon addition of an aromatic residue is due to an enhancement in binding. The fact that both moderately to highly active de-

rivatives (DOI-Gly-Phe, DOI-Gly-Phe-Phe, and DOI-Gly-Phe-Phe-Tyr) and the minimally active derivatives (DOI and DOI-Gly) will stimulate full biological effect in sufficient quantities also implies that the potency of a derivative is a direct result of its capacity to bind to the insulin receptor.

It was anticipated that one or more of the derivatives or intermediates would bind to the receptor in a nonproductive mode and thus act as an anti-insulin or insulin inhibitor. This behavior was not observed, and other insulin analogues are being prepared in an effort to investigate this possibility.

This leads to the conclusion that the B24–B26 region is absolutely required for correct binding of insulin to its receptor. The reduced activity of a human insulin with a mutation in this region (Tager et al., 1980; Gattner et al., 1980), as well as the invariance of this sequence in insulin from diverse species (Dayhoff, 1972), further supports this notion. An aromatic region is ideally suited to act as a binding determinant. The hydrophobic contours of an aromatic region are relatively rigid and not perturbed by solvent interactions, as a hydrophilic environment would be. Further, the system would be more specific than recognition based on a charged region. This would result in a more tightly controlled response, as is needed in a hormonal system. Our results indicate that this is indeed the case with insulin.

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Registry No. Bovine insulin, 11070-73-8; desoctapeptide-insulin, 52499-32-8; di-Boc-DOI-NHNH-C_6H_5, 79804-78-7; di-Boc-DOI, 76688-08-9; Z-Gly-Phe-OBu-*t*, 16881-36-0; Z-Phe-Phe-OBu-*t*, 13075-31-5; Z-Gly-Phe-Phe-OBu-*t*, 30225-19-5; Z-Gly-Phe-$NHNH_2$, 17942-42-6; Z-Phe-Tyr(Bu-*t*)-OBu-*t*, 84472-95-7; Z-Gly-Phe-Phe-Tyr(Bu-*t*)-OBu-*t*, 84498-66-8; Z-Gly-ONp, 1738-86-9; H-Phe-Phe-OBu-*t*·AcOH, 13106-11-1; Phe-Tyr(Bu-*t*)-OBu-*t*·HCl, 84472-96-8; Z-Gly-Phe-azide, 84472-97-9; Gly-Phe-Phe-Tyr(Bu-*t*)-OBu-*t*·HCl, 84472-98-0; di-Boc-DOI-phenyldiimide, 79804-77-6; DOI-Gly-Phe-Phe-Tyr, 84537-02-0; di-Boc-DOI-Gly-Phe-Phe-Tyr(Bu-*t*)-OBu-*t*, 84537-03-1; tri-Boc-insulin, 12584-75-7; DOI-Gly, 62169-17-9; DOI-Gly-Phe, 67016-79-9; DOI-Gly-Phe-Phe, 39302-19-7; DOI-Phe-Phe-Tyr, 84537-01-9.

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Isolation and Sequencing of an Active-Site Peptide from *Rhodospirillum rubrum* Ribulosebisphosphate Carboxylase/Oxygenase after Affinity Labeling with 2-[(Bromoacetyl)amino]pentitol 1,5-Bisphosphate†

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ABSTRACT: 2-[(Bromoacetyl)amino]pentitol 1,5-bisphosphate was reported to be a highly selective affinity label for ribulosebisphosphate carboxylase/oxygenase from *Rhodospirillum rubrum* [Fraij, B., & Hartman, F. C. (1982) *J. Biol. Chem. 257*, 3501–3505]. The enzyme has now been inactivated with a ^{14}C-labeled reagent in order to identify the target residue at the sequence level. Subsequent to inactivation, the enzyme was carboxymethylated with iodoacetate and then digested with trypsin. The only radioactive peptide in the digest was obtained at a high degree of purity by successive chromatography on DEAE-cellulose, SP-Sephadex, and Sephadex G-25. On the basis of amino acid analysis of the purified peptide, the derivatized residue was a methionyl sulfonium salt. Automated Edman degradation confirmed the purity of the labeled peptide and established its sequence as Leu-Gln-Gly-Ala-Ser-Gly-Ile-His-Thr-Gly-Thr-Met-Gly-Phe-Gly-Lys-Met-Glu-Gly-Glu-Ser-Ser-Asp-Arg. Cleavage of this peptide with cyanogen bromide showed that the reagent moiety was covalently attached to the second methionyl residue. Sequence homology with the carboxylase/oxygenase from spinach indicates that the lysyl residue immediately preceding the alkylated methionine corresponds to Lys-334, a residue previously implicated at the active site.

We recently reported the synthesis of BrAcNH-pentitol-P_2[1] (an epimeric mixture of the *ribo-* and *arabino*-pentitol derivatives) and showed that it behaves as an affinity label for ribulose-P_2 carboxylase/oxygenase from *Rhodospirillum rubrum*. On the basis of reversibility of inactivation by thiolysis and on the basis of the presence of carboxymethylhomocysteine in acid hydrolysates of the modified enzyme, we concluded that the reaction product is a sulfonium salt of methionine (Fraij & Hartman, 1982). In this report, we describe isolation of the tryptic peptide that contains the reagent moiety and confirm that a methionyl residue is the site of derivatization. Sequence analyses of the purified peptide reveal a homology with an active-site region of the corresponding spinach carboxylase.

Experimental Procedures

Materials

Bicine, ATP, NADH, glutathione, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate phosphokinase, and glycerophosphate dehydrogenase/triose phosphate isomerase were products of Sigma Chemical Co. TPCK-treated trypsin

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[1] Abbreviations: BrAcNH-pentitol-P_2, 2-[(bromoacetyl)amino]pentitol 1,5-bisphosphate; TPCK, *N*-tosylphenylalanine chloromethyl ketone; ribulose-P_2, D-ribulose 1,5-bisphosphate; carboxyribitol-P_2, 2-carboxyribitol 1,5-bisphosphate; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; SP, sulfopropyl.

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