

# A Biologically Active Insulin Analogue with Modification in the A<sup>2</sup> Position. [2-Valine-A] Sheep Insulin<sup>1,2)</sup>

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The synthesis and biological evaluation of [2-Valine-A] insulin ([Val<sup>2</sup>-A]insulin) is reported. In this insulin, the isoleucine residue in position A<sup>2</sup>, invariant in the majority of mammalian insulins, is substituted by valine. The same substitution, along with four others, occurs naturally in the insulin produced by the owl monkey. Owl monkey insulin exhibits *ca.* 20% of the activity of porcine insulin in *in vitro* insulin assays using human cells in culture. [Val<sup>2</sup>-A]insulin displays 20-22% of the activity of bovine insulin in *in vitro* insulin assays using rat liver plasma membranes or isolated rat adipocytes. We suggest that the substitution of valine for isoleucine at position A<sup>2</sup> is responsible for all or most of the diminution in potency of owl monkey insulin relative to porcine insulin. The data are discussed with regard to previous findings with insulin analogues in which isoleucine A<sup>2</sup> was replaced with norleucine, glycine and alanine.

**Keywords** owl monkey insulin; insulin analogue; insulin synthesis; insulin activity; receptor binding assay; lipogenesis assay; radioimmunoassay

Seino *et al.*<sup>3)</sup> have reported that the insulin isolated from the new world primate *Aotus trivirgatus*, (the owl, or night monkey), differs from porcine insulin in five amino acid residues, and that this insulin displays *ca.* 20% of the activity of porcine insulin in displacing <sup>125</sup>I-labelled insulin from insulin receptors in cultured cells. Owl monkey insulin also displays about 1% of the reactivity of porcine insulin in radioimmunoassay.<sup>3)</sup> Among the amino acid substitutions which distinguish porcine insulin from owl monkey insulin is the replacement of isoleucine at position 2 in the A chain, an invariant residue in most mammalian insulins.<sup>4)</sup> We have previously examined the effect of substituting three amino acid residues in position 2 of the A chain of insulin, norleucine,<sup>5)</sup> glycine and alanine.<sup>6)</sup> All of these insulin analogues were full agonists, but their potency relative to natural insulin was very low, ranging from 0.05% for [Gly<sup>2</sup>-A] insulin to 0.9% for [Nle<sup>2</sup>-A]insulin. These observations, together with similar results obtained with [Leu<sup>19</sup>-A]insulin,<sup>7)</sup> led us to propose that the interaction between the side-chains of isoleucine A<sup>2</sup> and tyrosine A<sup>19</sup>, which lie in van der Waals contact in the X-ray model of insulin,<sup>8)</sup> may be among the most crucial structural features contributing to the biological activity of insulin.<sup>7)</sup> In the present work, we demonstrate that the substitution of valine for isoleucine in position A<sup>2</sup> of insulin reduces the biological

activity of the resulting analogue relative to natural insulin, but that this reduction is rather modest in comparison to the reduction in potency caused by the substitution of glycine, alanine or norleucine in that position. In fact, the biological activity of [Val<sup>2</sup>-A]insulin in receptor binding assays is very similar to that of owl monkey insulin, which suggests that the natural substitution of valine for isoleucine in owl monkey insulin may explain all or most of its observed reduced potency relative to porcine insulin.<sup>3)</sup>

This work in conjunction with our studies involving the aforementioned substitutions at position A<sup>2</sup> of insulin,<sup>5,6)</sup> indicates that an amino acid residue at that position must possess, at minimum, a branched alkyl side chain of at least three carbon atoms to ensure proper interaction with the side chain of A<sup>19</sup> Tyr. We have previously proposed that this interaction is a stringent requirement for the maintenance of a conformation commensurate with high biological activity.<sup>7)</sup>

The synthesis of [Val<sup>2</sup>-A]insulin was carried out by the interaction of the S-sulfonated bovine (sheep) B chain with the S-sulfonated sheep [Val<sup>2</sup>]A chain [7] either by the procedure described previously<sup>9)</sup> or by the method of Chance *et al.*<sup>10)</sup> The synthesis of the S-sulfonated [Val<sup>2</sup>]A chain, patterned after our synthesis of insulin A chain analogues (*e.g.* Chu *et al.*),<sup>11)</sup> involved as the key in-

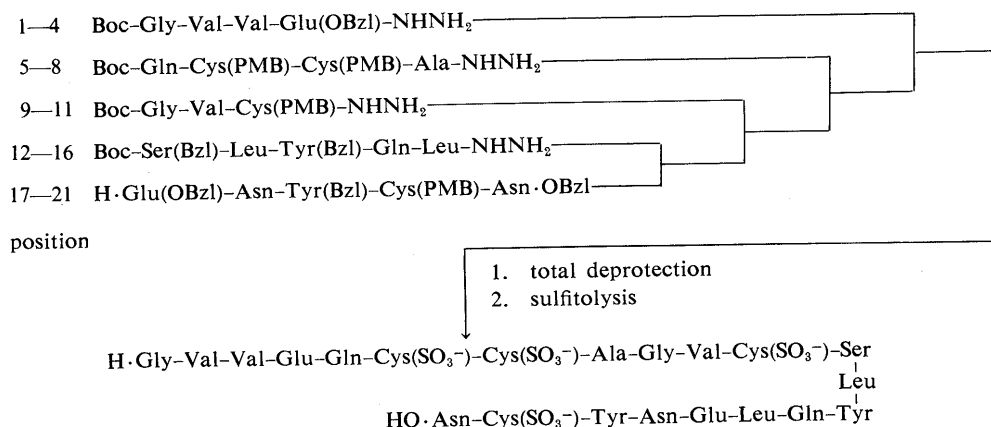


Fig. 1. Synthetic Route to Sheep [Val<sup>2</sup>]A Chain S-Sulfonate

intermediate the construction of the protected heneicosapeptide containing the entire amino acid sequence of that moiety. Its synthesis was accomplished by the fragment condensation approach using the azide method as the coupling procedure and is shown schematically in Fig. 1. We have selected five fragments as building blocks to accomplish the synthesis of the protected heneicosapeptide. The  $\alpha$ -amino function of intermediates was protected by the Boc group, whereas the secondary functions were protected with groups removable by trifluoromethanesulfonic acid.<sup>12)</sup> The C-terminal decapeptide fragment [1] (sequence 12–21) was prepared by the condensation of two pentapeptide units<sup>13,14)</sup> (sequences 12–16 and 17–21) and, after TFA–AcOH treatment, was interacted with the adjacent tripeptide, Boc–Gly–Val–Cys(PMB)–NHNH<sub>2</sub><sup>15)</sup> (sequence 9–11) to yield the C-terminal tridecapeptide [2] (sequence 9–21). Treatment of the latter compound with TFA–AcOH and coupling of the resulting product with the tetrapeptide Boc–Gln–Cys(PMB)–Cys(PMB)–Ala–NHNH<sub>2</sub><sup>15)</sup> (sequence 5–8) afforded the C-terminal heptadecapeptide [3] (sequence 5–21). In a final step, the TFA/AcOH-treated heptadecapeptide [3] was condensed with the N-terminal tetrapeptide [6] (sequence 1–4) to yield the protected heneicosapeptide containing the entire sequence of the [Val<sup>2</sup>]A chain. This product was deblocked on exposure<sup>12,15)</sup> to trifluoromethanesulfonic acid in the presence of thioanisole and *m*-cresol and the resulting deblocked peptide was converted to the S-sulfonated form using the procedure we have described previously.<sup>15)</sup> The crude product was purified by chromatography on a Sephadex G-15 column using 0.015 M NH<sub>4</sub>HCO<sub>3</sub> as the eluting solvent and then by chromatography on a Cellex-E column using 0.1 M Tris–HCl buffer and a linear NaCl gradient as described previously.<sup>11)</sup>

The purified sheep [Val<sup>2</sup>]A chain S-sulfonate was combined<sup>9,10)</sup> with sheep (bovine)B chain S-sulfonate and the resulting sheep [Val<sup>2</sup>-A] insulin was isolated<sup>9)</sup> from the combination mixture by chromatography on a Whatman CM 52 column equilibrated and eluted with a urea–acetate buffer (0.04 M sodium acetate in 8 M urea, pH 4.0). The insulin analogue was isolated from the column effluent as the hydrochloride *via* picrate.<sup>9,16)</sup> Chromatography of this product on a Sephadex G-50 column equilibrated and eluted with 1 M AcOH afforded the analogue in a highly purified form, as evidenced by reversed-phase HPLC and amino acid analysis after acid hydrolysis.

In receptor-binding assays in rat liver plasma membranes and in lipogenesis assays in isolated rat adipocytes [Val<sup>2</sup>-A]insulin exhibited potencies of  $21.7 \pm 5.4\%$  and  $23.4 \pm 4\%$  respectively relative to bovine insulin. In radioimmunoassay, the analogue displayed a potency of  $26.0 \pm 2.8\%$  relative to bovine insulin, substantially higher than the potency in radioimmunoassay of owl monkey insulin. This presumably reflects the fact that owl monkey insulin differs from porcine insulin at four positions other than A<sup>2</sup>, distributed throughout its primary structure, and thus able to affect immunogenic determinants other than those affected by the single substitution of valine for isoleucine in the present analogue.

## Experimental

Details of the analytical procedures used are given in a previous

publication.<sup>7)</sup> The homogeneity of all the intermediate peptide derivatives as ascertained by thin-layer chromatography on 6060 silica gel (Eastman Chromatogram Sheet) in the solvent systems chloroform–methanol–water (80:30:10 and 90:20:2). Amino acid analyses were carried out on samples that had been hydrolyzed with 6 N HCl under nitrogen in the presence of phenol for 24 h at 110 °C except as otherwise indicated, and were recorded on a Beckman High Performance Analyzer System 6300.

For insulin receptor binding assays, a fraction enriched in plasma membranes was prepared by differential centrifugation from the livers of fasted rats essentially as described previously.<sup>17)</sup> For lipogenesis assays fat adipocytes were obtained by collagenase digestion of fat pads obtained from rats fed *ad libitum*. Details of these assay procedures, including sources for material were described in a previous publication.<sup>6)</sup> Radioimmunoassays were performed using antibodies obtained from Arnel Products, New York, New York, U.S.A. A double antibody method was used employing guinea pig antibodies raised against porcine insulin, precipitated by rabbit anti-guinea pig serum. Further details have been described previously.<sup>6)</sup>

**Boc–Ser(Bzl)–Leu–Tyr(Bzl)–Gln–Leu–Glu(OBzl)–Asn–Tyr(Bzl)–Cys(PMB)–Asn–OBzl [1]** A solution of Boc–Glu(OBzl)–Asn–Tyr(Bzl)–Cys(PMB)–Asn–OBzl<sup>13)</sup> (2.5 g, 2.2 mmol) in a mixture of TFA–AcOH (21 ml–6 ml) was stored at room temperature for 2 h and concentrated under reduced pressure. Addition of ether caused precipitation of the product which was collected and dried. To a solution of this product in DMF (40 ml) and TEA (0.32 ml) was added the azide prepared from Boc–Ser(Bzl)–Leu–Tyr(Bzl)–Gln–Leu–NHNH<sub>2</sub><sup>14)</sup> (3.1 g, 3.5 mmol) and *tert*-butyl nitrite (0.48 ml) as described previously.<sup>14)</sup> After 48 h at 4 °C the reaction mixture was poured into a mixture of AcOEt (80 ml) and 5% citric acid solution (100 ml) and the precipitated product was collected, dried and precipitated from DMF and EtOH; yield 3.51 g (82%), mp 254–256 °C,  $[\alpha]_D^{25} -17.8^\circ$  ( $c=1.0$  DMSO). Amino acid ratios in a 6 N HCl hydrolysate: Asp<sub>1.9</sub>, Ser<sub>0.9</sub>, Glu<sub>2.0</sub>, Leu<sub>2.1</sub>, Tyr<sub>1.9</sub>. S-PMB–Cys was not determined. *Anal.* Calcd for C<sub>102</sub>H<sub>125</sub>N<sub>13</sub>O<sub>22</sub>S: C, 63.9; H, 6.62; N, 9.5. Found: C, 63.9; H, 6.74; N, 9.7.

**Boc–Gly–Val–Cys(PMB)–Ser(Bzl)–Leu–Tyr(Bzl)–Gln–Leu–Glu(OBzl)–Asn–Tyr(Bzl)–Cys(PMB)–Asn–OBzl [2]** The azide [prepared from 0.67 g (1.3 mmol) of Boc–Gly–Val–Cys(PMB)–NHNH<sub>2</sub><sup>15)</sup> and 0.19 ml *tert*-butyl nitrite] in DMF (15 ml) was added to an ice-chilled solution of TFA/AcOH-treated compound [1] (0.87 g, 0.45 mmol) in DMF (30 ml) containing TEA (0.63 ml). The reaction mixture was processed as described in the synthesis of compound [1]; yield 1.0 g (96%), mp 262–264 °C (dec),  $[\alpha]_D^{25} -15.1^\circ$  ( $c=1.0$ , DMSO). Amino acid ratios in an acid hydrolysate: Asp<sub>2.0</sub>Ser<sub>0.8</sub>Glu<sub>2.0</sub>Gly<sub>0.8</sub>Val<sub>0.7</sub>Leu<sub>2.0</sub>Tyr<sub>1.8</sub>. S-PMB–Cys was not determined. *Anal.* Calcd for C<sub>120</sub>H<sub>150</sub>N<sub>16</sub>O<sub>26</sub>S<sub>2</sub>: C, 62.7; H, 6.63; N, 9.8. Found: C, 62.6; H, 6.74; N, 9.8.

**Boc–Gln–Cys(PMB)–Cys(PMB)–Ala–Gly–Val–Cys(PMB)–Ser(Bzl)–Leu–Tyr(Bzl)–Gln–Leu–Glu(OBzl)–Asn–Tyr(Bzl)–Cys(PMB)–Asn–OBzl [3]** The azide [prepared from Boc–Gln–Cys(PMB)–Cys(PMB)–Ala–NHNH<sub>2</sub><sup>15)</sup> (1.56 g, 0.57 mmol)] in DMF (40 ml) was added to an ice-chilled solution of TFA/AcOH-treated compound [2] (0.96 g, 0.41 mmol) in DMF (40 ml) containing TEA (0.08 ml). The reaction mixture was processed as described in the synthesis of compound [1]; yield 1.01 g (83%), mp > 270 °C,  $[\alpha]_D^{25} -24.8^\circ$  ( $c=1.0$  DMSO). Amino acid ratios in an acid hydrolysate: Asp<sub>2.2</sub> Ser<sub>1.0</sub>Glu<sub>3.0</sub>Gly<sub>1.0</sub>Ala<sub>1.0</sub>Val<sub>0.7</sub>Leu<sub>2.0</sub>Tyr<sub>1.9</sub>. S-PMB–Cys was not determined. *Anal.* Calcd for C<sub>150</sub>H<sub>189</sub>N<sub>21</sub>O<sub>33</sub>S<sub>4</sub>: C, 61.2; H, 6.54; N, 10.0. Found: C, 61.0; H, 6.71; N, 10.0.

**Boc–Val–Val–Glu(OBzl)–NHNH–Troc[4]** A solution of Boc–Val–Glu(OBzl)–NHNH–Troc<sup>15)</sup> (12.52 g, 30 mmol) in TFA–anisole (24 ml–2 ml) was stored at room temperature for 45 min and was concentrated under reduced pressure. The remaining oily product was dissolved in toluene (50 ml) and this solution was concentrated under reduced pressure to dryness. The residue was washed with petroleum ether and dried over KOH *in vacuo*. To an ice-chilled solution of this product in DMF (30 ml), neutralized with TEA, was added a solution of Boc–Val–OH (5.43 g, 25 mmol) in DMF (20 ml) pre-activated for 3 h at 0 °C with DCC (6.19 g, 30 mmol) and HOBT (4.05 g, 30 mmol). After 24 h at room temperature the mixture was filtered and the filtrate diluted with AcOEt (300 ml). This solution was washed (1 N NH<sub>4</sub>OH, 10% citric acid and H<sub>2</sub>O), dried and concentrated to dryness. Upon trituration of the residue with petroleum ether the product solidified and reprecipitated from EtOH by the addition of ether and petroleum ether; yield 13.78 g (95%), mp 202–203 °C,  $[\alpha]_D^{25} -37.6^\circ$  ( $c=1.0$ , DMF). Amino acid ratios in a 6 N HCl hydrolysate (6 h at 150 °C): Glu<sub>1.0</sub>Val<sub>1.9</sub>. *Anal.* Calcd for C<sub>30</sub>H<sub>44</sub>N<sub>5</sub>O<sub>9</sub>Cl<sub>3</sub>: C, 49.7; H, 6.12; N, 9.7. Found: C, 49.8; H, 5.94; N, 9.5.

**Boc–Gly–Val–Val–Glu(OBzl)–NHNH–Troc [5]** A solution of Boc–

Gly-OH (5.26 g, 30 mmol) in DMF (10 ml), pre-activated for 3 h at 0°C, with DCC (7.43 g, 36 mmol) at HOBT (4.86 g 36 mmol) was mixed with a solution of a TFA/anisole-treated compound [4] (13.0 g, 18 mmol) in DMF (30 ml) neutralized with TEA (*ca.* 3.5 ml). The reaction mixture was processed as described above and the product purified by precipitation from ether-petroleum ether; yield 11.97 g (85%), mp > 265°C,  $[\alpha]_D^{25}$  -24.9° (*c* = 1, DMF). Amino acid ratios in a 6N HCl hydrolysate (6 h at 150°C); Glu<sub>1.0</sub>Gly<sub>1.0</sub>Val<sub>1.9</sub>. Anal. Calcd for C<sub>32</sub>H<sub>47</sub>Cl<sub>3</sub>N<sub>6</sub>O<sub>10</sub>: C, 49.1; H, 6.12; N, 10.7. Found: C, 49.3; H, 5.94; N, 10.7.

**Boc-Gly-Val-Val-Glu(OBzl)-NHNH<sub>2</sub> [6]** The protected tetrapeptide derivative [5] (1.77 g, 2.26 mmol) in DMF-AcOH (50 ml-10 ml) was treated with Zn powder (4 g, 61 mmol) for 4 h and the solution was filtered (with the aid of Celite). The filtrate was concentrated to a small volume and then mixed with a saturated solution of EDTA disodium salt (5 ml). After 30 min, the mixture was adjusted to pH 8 with 5% NaHCO<sub>3</sub> and the precipitated product was collected, washed, dried and reprecipitated from DMF-H<sub>2</sub>O; yield 1.0 g (73%), mp > 270°C,  $[\alpha]_D^{25}$  -7.0° (*c* = 1.0, DMF). Amino acid ratios in an acid hydrolysate: Glu<sub>1.0</sub>Gly<sub>0.9</sub>Val<sub>1.5</sub>. Anal. Calcd for C<sub>29</sub>H<sub>46</sub>N<sub>6</sub>O<sub>8</sub> · 1/2 H<sub>2</sub>O: C, 56.6, H, 7.74; N, 13.6. Found: C, 56.3; H, 7.65; N, 13.2.

**H-Gly-Val-Val-Glu-Gln-Cys(SO<sub>3</sub><sup>-</sup>)-Cys(SO<sub>3</sub><sup>-</sup>)-Ala-Gly-Val-Cys(SO<sub>3</sub><sup>-</sup>)-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys(SO<sub>3</sub><sup>-</sup>)-Asn-OH Sheep Insulin [Val<sup>2</sup>]A Chain S-Sulfonate [7]** The azide prepared from compound [6] (0.7 g, 1.1 mmol) in the usual way in DMF (30 ml) was added to an ice-chilled solution of a TFA/AcOH-treated sample of compound [3] (0.7 g, 0.24 mmol) in DMF (45 ml) containing TEA (0.034 ml, 0.24 mmol). After 48 h at 4°C, the mixture was diluted with AcOEt (80 ml) and 5% citric acid (100 ml) and the precipitated crude protected heneicosapeptide was collected, washed with EtOH and reprecipitated from DMF-H<sub>2</sub>O; yield 0.58 g. Total deprotection of this product was carried out by a slight modification of the procedure of Yajima *et al.*<sup>12)</sup> as described previously,<sup>15)</sup> and conversion of the resulting reduced product to the S-sulfonated form was performed in the usual way.<sup>15)</sup> Briefly, a solution of the above protected heneicosapeptide (0.2 g) in 1 M trifluoromethanesulfonic acid in TFA (4.9 ml), thioanisole (0.68 ml) and *m*-cresol (0.61 ml) was stored at 0°C for 2 h. To this solution, cooled to -10°C, was then added dropwise and with vigorous stirring 8 M guanidine hydrochloride (25 ml) containing concentrated NH<sub>4</sub>OH (5 ml). The mixture was extracted with ether (3 × 50 ml) and to the aqueous layer, adjusted to pH 8.9 with NH<sub>4</sub>OH were added sodium sulfite (1.13 g) and freshly prepared sodium tetrathionate<sup>18)</sup> (0.54 g). After 3.5 h at room temperature, the mixture was placed in Spectrapor membrane tubing No. 3 and dialyzed against four changes of

distilled water (4 l each) at 4°C for 24 h. Lyophilization of the dialysate afforded the crude [Val<sup>2</sup>]A chain S-sulfonate. This product was dissolved in 0.015 M NH<sub>4</sub>HCO<sub>3</sub> (10 ml) and chromatographed on a Sephadex G-15 column (4.2 × 45 cm) equilibrated and eluted with 0.015 M NH<sub>4</sub>HCO<sub>3</sub>. The effluent corresponding to the main peak, as monitored with an ISCO spectrophotometer was lyophilized and the [Val<sup>2</sup>]A chain S-sulfonate was obtained as a white powder; yield 125 mg (82.4% based on the protected heneicosapeptide).

For purification this product (60 mg) was dissolved in 0.1 M Tris-HCl buffer (pH 7; 3 ml) and applied on a Cellex-E column (1.2 × 42 cm) equilibrated with the same buffer. Elution of the column was carried out with a linear NaCl gradient formed by adding to the above buffer (250 ml) 0.6 M NaCl in the same buffer (250 ml).<sup>14)</sup> The chromatographic pattern as monitored by an ISCO spectrophotometer and a conductivity meter (Radiometer, Copenhagen), is shown in Fig. 2a. The effluent corresponding to the main peak (255–305 ml) was collected, dialyzed as described above and lyophilized to a white powder; yield 21 mg. The rest of the material was similarly purified; total yield, 42 mg (27.8% based on the protected heneicosapeptide). Upon rechromatography of this material (42 mg) on the same Cellex-E column and under identical conditions, the pattern shown in Fig. 2b was obtained. From the effluent (210–310 ml) after dialysis and lyophilization, purified [Val<sup>2</sup>]A chain S-sulfonate was obtained as a white fluffy powder; yield 37.5 mg (24.8% overall, based on the protected heneicosapeptide). Amino acid ratios in an acid hydrolysate: Asp<sub>2.1(2)</sub>Ser<sub>1.0(1)</sub>Glu<sub>3.8(4)</sub>Gly<sub>1.8(2)</sub>Ala<sub>1.0(1)</sub>Val<sub>1.7(3)</sub>Leu<sub>2.1(2)</sub>Tyr<sub>2.0(2)</sub>. Cysteine was not determined. This material was completely digested by aminopeptidase M.

**S-Sulfonated Derivative of the B Chain of Sheep Insulin** The B chain of sheep insulin is identical to the corresponding chain of bovine insulin.<sup>19,20)</sup> The bovine (sheep) insulin B chain S-sulfonate was prepared by sulfityolysis of bovine insulin followed by CM-cellulose chromatography of the resulting S-sulfonated A- and B-chains as described previously,<sup>21)</sup> with the only difference that the sulfityolysis was carried out for 3 h instead of 24 h.

**Synthesis and Isolation of Sheep [Val<sup>2</sup>-A]Insulin** The synthesis of this analogue was carried out by the interaction of the S-sulfonated [Val<sup>2</sup>]sheep A chain with the S-sulfonated sheep (bovine) B chain by the procedure described previously,<sup>9)</sup> or by the procedure of Chance *et al.*<sup>10)</sup> A typical experiment according to the second route is as follows. A solution of

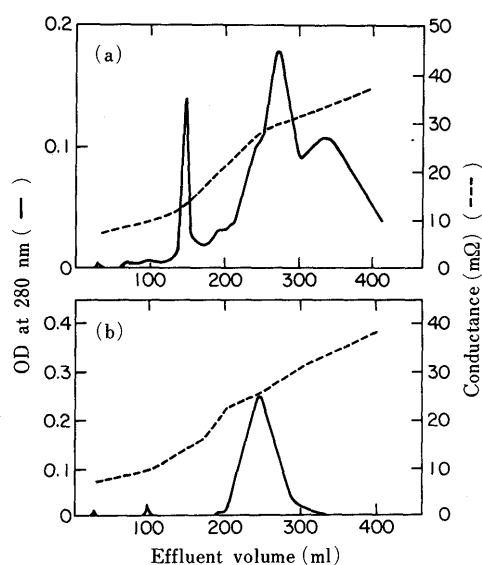


Fig. 2. (a) Chromatography of [Val<sup>2</sup>]A Chain S-Sulfonate on a 1.2 × 42 cm Cellex-E Column with 0.1 M Tris-HCl Buffer and a Linear NaCl Gradient

The column was monitored by an ISCO spectrophotometer and a conductivity meter.

(b) Rechromatography of the Product Obtained after Dialysis and Lyophilization of the Main Peak Effluent (255–305 ml) Depicted in Panel (a)

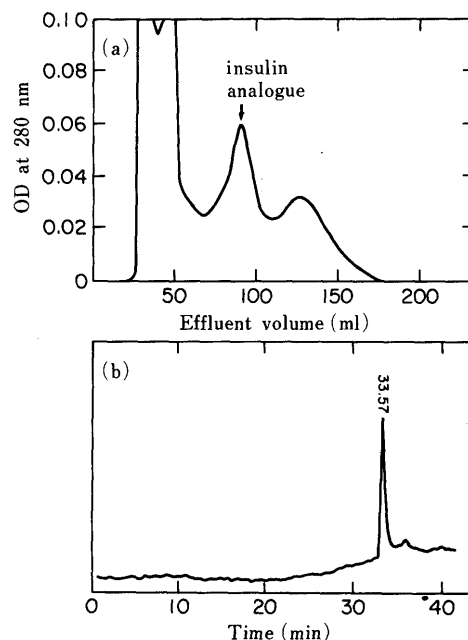


Fig. 3. (a) Chromatography of a Combination Mixture (See Experimental Section) of the S-Sulfonates of Sheep [Val<sup>2</sup>]A Chain and Sheep (Bovine) B Chain on a CM-Cellulose Column (1.2 × 39 cm) with Urea-Acetate Buffer (0.04 M Sodium acetate-8 M Urea, pH 4.0)

The column was monitored with an ISCO Spectrophotometer. The [Val<sup>2</sup>-A]insulin (80–110 ml of effluent) was eventually recovered as the hydrochloride.

(b) HPLC of Synthetic [Val<sup>2</sup>-A]Insulin on a 0.45 × 25 cm Vydac 218 TP Column at 0.5 ml/min with a 10–50% Linear Gradient of 2-Propanol in 0.1% TFA over 70 min

[Val<sup>2</sup>]A chain S-sulfonate (37 mg) and sheep B chain S-sulfonate (18.5 mg) in 0.1 M glycine buffer (pH 10.5; 10 ml) containing dithiothreitol (13 mg) was stirred at 4°C for 24 h and then processed as described previously.<sup>9</sup> Isolation of the insulin analogue from the combination mixture was achieved by chromatography on a CM-cellulose column with 8 M urea-acetate buffer (0.04 M sodium acetate-8 M urea, pH 4.0) and applied to a Whatman CM 52 column (1.2 × 39 cm) equilibrated and eluted with the same buffer. The chromatographic pattern, as monitored with an ISCO spectrophotometer, is shown in Fig. 3a. The effluent containing the insulin analogue (80–110 ml) was collected and dialyzed (0°C) for 24 h against four 4-l changes of distilled water (Spectrapor membrane tubing No. 3). The dialysate was then concentrated to a small volume *in vacuo* and the analogue was isolated *via* picrate as the hydrochloride.<sup>9,16</sup> In a final purification step this product was chromatographed on a Sephadex G-50 column (0.9 × 24 cm) equilibrated and eluted with 1 M AcOH. The effluent under the main peak (10–20 ml), was collected and lyophilized to a white fluffy material; yield 1 mg. On reversed-phase HPLC using a Vydac 218 TP column (0.45 × 25 cm) connected to a Beckman liquid chromatography system, with a 10–50% linear gradient of 2-propanol in 0.1% TFA over 70 min (flow rate: 0.5 ml/min) the purified insulin analogue gave the pattern shown in Fig. 3b. Amino acid ratios in an acid hydrolysate: Asp<sub>3.1(3)</sub> Thr<sub>0.9(1)</sub> Ser<sub>2.0(2)</sub> Pro<sub>1.2(1)</sub> Glu<sub>7.0(7)</sub> Gly<sub>5.0(5)</sub> Ala<sub>3.2(3)</sub> Val<sub>4.7(6)</sub> Leu<sub>5.6(6)</sub> Tyr<sub>3.5(4)</sub> Phe<sub>2.5(3)</sub> Lys<sub>1.1(1)</sub> His<sub>2.0(2)</sub> Arg<sub>1.2(1)</sub>. Cysteine was not determined.

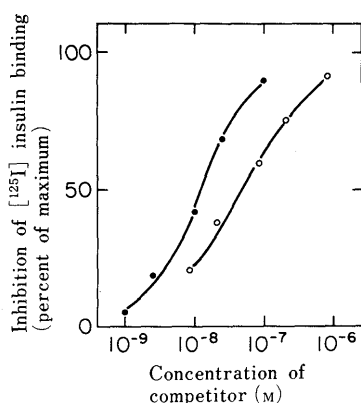


Fig. 4. Effect of Bovine Insulin (●) and [Val<sup>2</sup>-A]Insulin (○) on the Binding of <sup>125</sup>I-Insulin to Insulin Receptors in Rat Liver Plasma Membranes

The inhibition of binding of labelled insulin, expressed as per cent of maximum, is presented as a function of the concentration of competing compound. Non-specific binding has been subtracted from all values. The data points represent the means of triplicate determinations in a representative experiment which was performed three times. In this experiment, specific binding in the absence of competitor amounted to 10.2% of the input radioactivity.

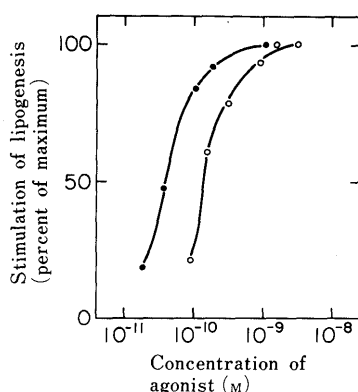


Fig. 5. Effect of Bovine Insulin (●) and [Val<sup>2</sup>-A]Insulin (○) on the Stimulation of Lipogenesis in Isolated Rat Adipocytes

The stimulation of the conversion of 3-[<sup>3</sup>H]glucose into organic-extractable form, expressed as percent of maximum, is presented as a function of the concentration of stimulatory compound. The data points represent the means of triplicate determinations in a representative experiment which was performed three times. In this experiment, zero and 100% stimulation refer respectively to 6.9 and 57.3 nmol of glucose per mg dry weight of cells.

**Biological Evaluation of [Val<sup>2</sup>-A] Sheep Insulin** [Val<sup>2</sup>-A]insulin was compared with bovine insulin in three types of assays: 1) receptor binding in rat liver plasma membranes, in which relative potency is calculated from the ratio of the concentrations of natural insulin and insulin analogue required to displace 50% of specifically bound <sup>125</sup>I-insulin; 2) lipogenesis, in which relative potency is calculated from the ratio of concentrations of bovine insulin and analogue required to achieve 50% of the maximum stimulation of incorporation of 3-<sup>3</sup>H-glucose into organic-extractable material by isolated rat adipocytes; and 3) radioimmunoassay, in which the ability of natural insulin and analogue are compared for their ability to compete with <sup>125</sup>I-insulin in binding to guinea-pig antibodies raised against porcine insulin. In radioimmunoassay, the data are expressed as described by Hales and Randle,<sup>22</sup> and relative potency is obtained by comparing the slopes of the resulting straight lines.

Figure 4 shows the behavior of [Val<sup>2</sup>-A]insulin in receptor-binding assays in rat liver plasma membranes. Competition with <sup>125</sup>I-insulin is concentration dependent, the dose-response curves are essentially parallel, and the calculated potency of the analogue is 21.7 ± 5.4% relative to bovine insulin. The effect of [Val<sup>2</sup>-A]insulin and bovine insulin upon lipogenesis is shown in Fig. 5. The analogue is a full agonist, reaching the same maximum stimulation as that seen with insulin, the dose-response curves are again parallel, and the calculated potency of the analogue is 23.4 ± 4.0% relative to bovine insulin, in good agreement with the potency displayed in receptor-binding assays. In radioimmunoassay (data not shown), [Val<sup>2</sup>-A]insulin displays a potency of 26.0 ± 2.8% relative to bovine insulin.

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#### References and Notes

- 1) This paper is dedicated to our distinguished colleague and friend, Professor Haruaki Yajima, on the occasion of his retirement from the Faculty of Pharmaceutical Sciences, Kyoto University, Japan.
- 2) Amino acids used in this investigation are of the L-configuration. The following abbreviations are used: Ac=acetyl, Bzl=benzyl, Boc=*tert*-butoxycarbonyl, CM=carboxymethyl, DCC=dicyclohexylcarbodiimide, DMF=dimethylformamide, DMSO=dimethyl sulfoxide, EDTA=ethylenediaminetetraacetic acid, HOBT=*N*-hydroxybenzotriazole, HPLC=high performance liquid chromatography, PMB=*p*-methoxybenzyl, TEA=triethylamine, TFA=trifluoroacetic acid, Tris=2-amino-2-(hydroxymethyl)-1,3-propanediol, Troc=2,2,2-trichloroethoxycarbonyl.
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