Possible Involvement of the A^{20}–A^{21} Peptide Bond in the Expression of the Biological Activity of Insulin. 2. [21-Asparagine diethylamide-A]insulin†

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ABSTRACT: We have synthesized [21-asparagine diethylamide-A]insulin, which differs from the parent molecule in that the free carboxyl group of the C-terminal amino acid residue, asparagine, of the A chain moiety has been converted to a diethylamide group. The analogue displays equivalent potency in receptor binding and biological activity, 48% and 56%, respectively, relative to bovine insulin. In contrast, we have reported previously [Burke, G. T., Chanley, J. D., Okada, Y., Cosmatos, A., Ferderigos, N., & Katsoyannis, P. G. (1980) *Biochemistry 19*, 4547–4556] that [21-asparaginamide-A]insulin exhibits a divergence in these properties, ca. 60% in receptor binding and ca. 13% in biological activity. The disparity in the biological behavior of these analogues is discussed, and we ascribe the modulation of biological activity independent of receptor binding activity observed between these analogues to the difference in the negativity of the carbonyl oxygen of the A chain moiety C-terminal amino acid residue.

We have previously reported the synthesis of an insulin analogue, [21-asparaginamide-A]insulin (Burke et al., 1980), that displays a divergence between receptor binding affinity (ca. 60%) and biological activity (ca. 13%) relative to bovine insulin. This finding implicated the C-terminal region of the A chain of insulin in modulating the biological activity of the hormone independent of binding affinity. It was thus of interest to explore the effects of a number of modifications of the C-terminal residue of the A chain of insulin on its biological activity. In this paper, we describe the synthesis and biological evaluation of sheep [21-asparagine diethylamide-A]insulin, in which the free carboxyl group of the C-terminal residue of the A chain, asparagine, has been converted into the diethylamide derivative.

Experimental Procedures and Results

Details of materials, analytic procedures, and biological evaluation (receptor binding, lipogenesis, and radioimmunoassay) are given in the preceding paper (Chu et al., 1987a).

General Aspects of Synthesis of Sheep [21-Asparagine diethylamide-A]insulin. As for the synthesis of the analogues described in the preceding paper (Chu et al., 1987a), the synthesis of this analogue was carried out by the combination of the S-sulfonated bovine (sheep) B chain with the S-sulfonated [21-asparagine diethylamide]A chain. The overall procedure for the synthesis of the latter compound is comparable to that outlined in the preceding paper (Chu et al., 1987a). Removal of the blocking groups from the protected chain (VIII)[1] prior to sulfitolysis was carried out on exposure to methanesulfonic acid as described previously (Ogawa et al., 1984).

Glycyl-L-*isoleucyl*-L-*valyl*-L-*glutamyl*-L-*glutaminyl*-*S*-*sulfo*-L-*cysteinyl*-*S*-*sulfo*-L-*cysteinyl*-L-*alanylglycyl*-L-*valyl*-*S*-*sulfo*-L-*cysteinyl*-L-*seryl*-L-*leucyl*-L-*tyrosyl*-L-*glutaminyl*-L-*leucyl*-L-*glutamyl*-L-*asparaginyl*-L-*tyrosyl*-*S*-*sulfo*-L-*cysteinyl*-L-*asparagine Diethylamide* (*Sheep Insulin [21-Asparagine diethylamide]A Chain S-Sulfonate*) (*IX*). A solution of the protected heneicosapeptide VIII (253 mg) in 4 M methanesulfonic acid in TFA containing thioanisole (1.5 mL) and *m*-cresol (1 mL) was stored for 20 min at 0 °C and for 1 h at room temperature. Subsequently, to this solution, cooled to −5 °C, was added dropwise and with vigorous stirring a mixture of 8 M guanidine hydrochloride (25 mL) and concentrated NH_4OH (4 mL). During this process, the temperature of the reaction mixture was kept below 5 °C. The resulting mixture (pH ~4) was extracted 3 times with ether (50 mL each), and to the aqueous layer, adjusted to pH 8.9 with NH_4OH, were added sodium sulfite (1.7 g) and sodium tetrathionate (0.8 g). The mixture was stirred at room temperature for 3.5 h and then 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 dialyzate afforded the crude chain analogue. For a preliminary purification, this material was dissolved in 0.015 M NH_4HCO_3 (5 mL) and chromatographed on a Sephadex G-15 column (4.5 × 45 cm) equilibrated and eluted with 0.015 M NH_4HCO_3 at a flow rate of ca. 130 mL/h. The effluent corresponding to the main peak, as monitored by an ISCO spectrophotometer, was lyophilized, and the S-sulfonated chain analogue was obtained as a white powder, weight 210 mg. This material (63 mg) was dissolved in 0.1 M Tris-HCl buffer (pH 7.0, 5 mL) and placed on a Cellex-E column (1.2 × 40 cm), which was equilibrated with the same buffer. Elution of the column with Tris-HCl buffer (pH 7.0) and a linear NaCl gradient as described previously (Chu et al., 1987a) gave the pattern shown in Figure 1. The eluate under the main peak (430–520 mL) was collected, dialyzed as above, and lyophilized, weight 26 mg. Rechromatography of this material (84 mg) on a Cellex-E column under identical conditions as described above gave the pattern shown in Figure 1. From the effluent (315–460 mL) after dialysis and lyophilization the purified sheep insulin [21-asparagine diethylamide]A chain *S*-sulfonate was obtained, weight 71 mg. On thin-layer electrophoresis, under the conditions mentioned in the pre-

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[1] Abbreviations: CM, carboxylmethyl; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; TEA, triethylamine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride. Compounds designated by Roman numerals are described fully in the supplementary material (see paragraph at end of paper regarding supplementary material).

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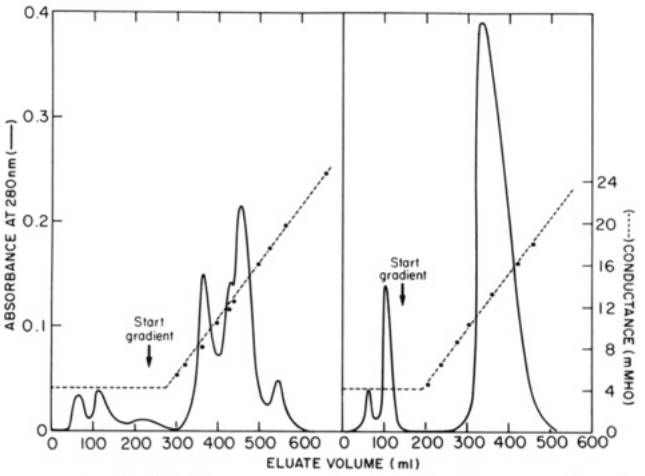

FIGURE 1: (Left) Chromatography of crude [21-asparagine diethylamide]A chain *S*-sulfonate on a 1.2 × 40 cm Cellex-E column with 0.1 M Tris-HCl buffer (pH 7.0) and a linear NaCl gradient. The effluent was monitored by an ISCO spectrophotometer and a conductivity meter (Radiometer, Copenhagen). (Right) Rechromatography of the product obtained after dialysis and lyophilization of the main peak effluent (430–520 mL) depicted in the left panel.

Table I: Amino Acid Composition[a] of an Acid Hydrolysate and an Enzymatic Digest (Leucine Aminopeptidase) of the S-Sulfonated [21-Asparagine diethylamide]A Chain

	acid hydrolysis		enzymatic hydrolysis	
amino acid	theory	found	theory	found
Asp	2	1.9	0	0
Ser	1	0.8	1	*b*
Asn	0	0	0	*b*
Gln	0	0	2	*b*
Glu	4	4	2	1.9
Gly	2	2	2	1.9
Ala	1	1	1	1
$^1/_2$-Cys	4	nd[c]	0	0
Val	2	1.9	2	1.8
Ile	1	0.8	1	0.9
Leu	2	2.1	2	2
Tyr	2	2	2	1.9
S-sulfo-Cys	0	0	4	4.1

[a] Number of amino acid residues per molecule. [b] Emerge on the same position and not determined. [c] nd, not determined.

ceding paper (Chu et al., 1987a), the synthetic chain analogue moved as a single component (data not shown). Amino acid analysis of the purified [21-asparagine diethylamide]A chain *S*-sulfonate after acid hydrolysis gave the molar ratios shown in Table I, in good agreement with the theoretically expected values. The synthetic chain was completely digested by leucine aminopeptidase as was shown by amino acid analysis of the digest (Table I).

S-Sulfonated Derivative of the B Chain of Sheep Insulin. This compound was prepared as indicated in the preceding paper (Chu et al., 1987a).

Synthesis and Isolation of Sheep [21-Asparagine diethylamide-A]insulin. The synthesis of this analogue by interaction of the respective S-sulfonated A and B chains and its isolation in purified form were carried out as described in the preceding paper (Chu et al., 1987a). A typical combination experiment consisted of 20 mg of [21-asparagine diethylamide]A chain *S*-sulfonate, 13 mg of S-sulfonated sheep B chain, and 3.4 mg of dithiothreitol in 0.1 M glycine buffer (4 mL, pH 10.5). Chromatography of the combination mixture (CM-cellulose column, acetate buffer, pH 3.3, and linear NaCl gradient) gave the pattern shown in Figure 2. The eluate containing the synthetic analogue (188–238 mL) was processed as described

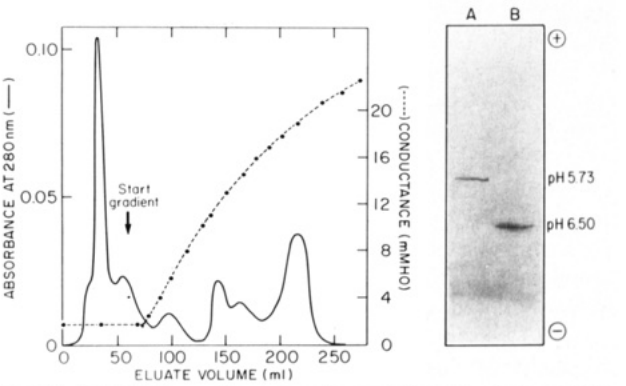

FIGURE 2: (Left) Chromatography of a combination mixture of the [21-asparagine diethylamide]A chain *S*-sulfonate with the S-sulfonated sheep insulin B chain on a 0.9 × 24 cm CM-cellulose column with acetate buffer (Na^+, 0.024 M, pH 3.3) and an exponential NaCl gradient. The effluent, monitored as in Figure 1, containing the synthetic analogue (188–238 mL) was recovered as the hydrochloride. (Right) Paper print of thin-layer isoelectric focusing of natural bovine insulin (A) and synthetic sheep [21-asparagine diethylamide-A]insulin (B) in a 1:1 mixture of pH 3–10 and pH 4–6 ampholytes on a 20-cm separation distance. Focusing: constant power; 8 W for 4 h.

Table II: Amino Acid Composition[a] of an Acid Hydrolysate of Sheep [21-Asparagine diethylamide-A]insulin

amino acid	theory	found	amino acid	theory	found
Lys	1	1	Gly	5	5.1
His	2	1.9	Ala	3	3
Arg	1	0.9	$^1/_2$-Cys	6	nd[b]
Asp	3	3.1	Val	5	4.7
Thr	1	0.8	Ile	1	0.8
Ser	2	2.2	Leu	6	6
Pro	1	1	Tyr	4	3.8
Glu	7	7	Phe	3	3

[a] Number of amino acid residues per molecule. [b] nd, not determined.

previously (Katsoyannis et al., 1967a,b), and the purified sheep [21-asparagine diethylamide-A]insulin was isolated via picrate as the hydrochloride (1.6 mg).

Amino acid analysis of the purified analogue after acid hydrolysis gave the molar ratios shown in Table II, in good agreement with the theoretically expected values. On isoelectric focusing, under the conditions described previously (Chu et al., 1987a), sheep [21-asparagine diethylamide-A]insulin focused in one band at pH 6.50, whereas bovine insulin in the same system focused at pH 5.73 (Figure 2).

Biological Evaluation of the Synthetic Insulin Analogue. [21-Asparagine diethylamide-A]insulin displayed a calculated potency of 48.4 ± 8.9% in receptor binding assays and 56.2 ± 2.2% in lipogenesis. These results represent the mean potency ± 1 SD obtained from at least three dose–response curves (not shown). In radioimmunoassay, the analogue displayed a potency of 21.8% (data not shown). All potency values are expressed as percentages of bovine insulin.

Discussion

A receptor binding region for insulin has been proposed that includes the following amino acid residues: A^1 Gly, A^{19} Tyr, A^{21} Asn, B^{12} Val, B^{13} Glu, B^{16} Tyr, B^{23} Gly, B^{24} Phe, and B^{25} Phe (Blundell et al., 1972; Blundell & Wood, 1975; Pullen et al., 1976). We have synthesized a number of insulin analogues in which several of the above residues have been singly altered. Biological evaluation of these analogues showed that where receptor binding affinity was decreased an equivalent decrease in biological potency was observed [e.g., Cosmatos et al. (1978) and Ferderigos et al. (1983)]. The striking exception was the behavior of [21-asparaginamide-A]insulin, a synthetic ana-

logue that exhibited divergence in receptor binding affinity and biological activity, ca. 60% and 13%, respectively, relative to natural bovine insulin (Burke et al., 1980). This finding implicated the carboxyl-terminal region of the insulin A chain in the putative "message region" of the hormone (Burke et al., 1980; Schwartz et al., 1983). Direct modification of the A^{21} amino acid residue of insulin, in which a free carboxyl group is maintained [Asn → Gly and Asn → β-Ala (unpublished data from this laboratory)], resulted in a modest reduction in biological activity (40–60% relative to natural insulin) but most importantly an equivalent reduction in receptor binding affinity. This suggests that the nature of the side chain per se of the A^{21} residue only modestly affects receptor binding and that it is not responsible for the modulation of biological activity of insulin analogues. However, as indicated above, amidation of the A^{21} carboxyl group led to [21-asparagin-amide-A]insulin, displaying a modest reduction in receptor binding affinity but a dramatic decrease in biological activity. In this paper, diethylamidation of the A^{21} carboxyl group results in an analogue, [21-asparagine diethylamide-A]insulin, with binding affinity comparable to [21-asparaginamide-A]-insulin, ca. 48%, but now with equivalent biological activity, ca. 56%, relative to insulin. In other words, the conversion of the A^{21} α-$CONH_2$ to α-$CON(C_2H_5)_2$ does not affect the binding affinity but dramatically increases the biological activity of the molecule. The immunological potency of this analogue is ca. 2.5-fold lower than is the biological activity. It was noted in the preceding paper (Chu et al., 1987a) that this is not surprising since the C-terminal region of the A chain of insulin appears to be an important immunogenic determinant (Arquilla et al., 1969).

As we discussed above, the A^{21}-modified insulins in which a free carboxyl group is maintained display receptor binding and biological potencies similar to those of the diethylamide analogue; thus, it is not likely that the increase in biological activity of the present analogue as compared to that of [21-asparaginamide-A]insulin is due directly to the diethylamide moiety. The conversion of the A^{21} $CONH_2$ to $CON(C_2H_5)_2$ alters the electron distribution of the amide moiety by the well-recognized electron-repelling inductive affect of the alkyl group [–C(O)–N(R)–R ↔ —$C(O^-)$=N^+(R)—R] and results in an increase in negative charge on the A^{21} carbonyl oxygen. It then appears reasonable to speculate that the increase of negative charge of the A^{21} carbonyl oxygen in the carboxydiethylamide moiety is sufficient to allow this group to play the same role as does the free carboxylate anion in this position in natural insulin and in the above-mentioned synthetic insulins, which maintain a free carboxyl group in position A^{21}. All of these molecules have the same intrinsic activity as natural insulin once bound to the receptor, and their modestly reduced biological activity as compared to natural insulin is wholly due to a reduction in binding affinity. Accordingly, the amidation of the free carboxyl group in [21-asparaginamide-A]insulin modulates the biological activity by sufficiently reducing the negative charge on the A^{21} carbonyl oxygen so as to result in a dissociation of biological potency from binding affinity for this analogue. Whether the intrinsic negativity of the A^{21} carbonyl oxygen is directly involved in the modulation of biological activity or is indirectly involved via an inductive field effect on a neighboring structural feature of the hormone molecule is not apparent from these data. In the preceding paper (Chu et al., 1987a), we have shown that the A^{20}–A^{21} amide bond is of considerable importance for the expression of biological activity in insulin. It is of interest to note that the penultimate peptide bond in the luteinizing hormone-releasing hormone (LH-RH) is similarly important for the expression of the biological activity of that molecule (Fujino et al., 1972; Coy et al., 1975). Furthermore, modifications that would be expected to alter the electronic state of the penultimate peptide bond of LH-RH affect its biological activity (Coy et al., 1975). In view of this and the above discussion, it is tempting to speculate that the electronic state (dipolar form) of the A^{20}–A^{21} peptide bond (-Cys-Asn), in the penultimate position of the A chain of insulin, influenced by an inductive field effect of the A^{21} carbonyl oxygen and properly oriented within the hormone molecule (Chu et al., 1987a) may be involved in the modulation of the biological activity of insulin. According to this hypothesis, the A^{20}–A^{21} peptide bond may be a significant element of the "message region" of insulin. In the following paper (Chu et al., 1987b), evidence is presented that strengthens this speculation.

Acknowledgments

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Supplementary Material Available

Complete synthetic details of compounds I–VIII including references (7 pages). Ordering information is given on any current masthead page.

Registry No. I, 110433-92-6; I (*p*-nitrophenyl ester), 4587-33-1; II, 110433-93-7; II (des-BOC), 110433-96-0; III, 110433-94-8; III (des-BOC), 110433-98-2; IV, 110433-97-1; IV (des-BOC), 110433-99-3; V, 110456-70-7; V (des-BOC), 110434-02-1; VI, 110434-01-0; VI (des-BOC), 110434-04-3; VII, 110456-71-8; VII (des-BOC), 110434-06-5; VII (des-BOC)·CF_3COOH, 110434-07-6; VIII, 110434-05-4; IX, 110456-73-0; BOC-*S*-*p*-$MeOC_6H_4CH_2$-Cys-O-*p*-$C_6H_4NO_2$, 53843-86-0; BOC-Asn-Tyr-$NHNH_2$, 96573-54-5; BOC-Asn-Tyr-N_3, 110433-95-9; BOC-γ-$C_6H_5CH_2O$-Glu-O-*p*-$C_6H_4NO_2$, 7536-59-6; BOC-Ser-Leu-Tyr-Gln-Leu-$NHNH_2$, 42547-33-1; BOC-Ser-Leu-Tyr-Gln-Leu-N_3, 110434-00-9; BOC-Gly-Val-*S*-*p*-$MeOC_6H_4CH_2$-Cys-$NHNH_2$, 96573-60-3; BOC-Gly-Val-*S*-*p*-$MeOC_6H_4CH_2$-Cys-N_3, 110434-03-2; BOC-Gln-*S*-*p*-$MeOC_6H_4CH_2$-Cys-*S*-$MeOC_6H_4CH_2$-Cys-Ala-$NHNH_2$, 96573-65-8; BOC-Gln-*S*-*p*-$MeOC_6H_4CH_2$-Cys-*S*-*p*-$MeOC_6H_4CH_2$-Cys-Ala-N_3, 110456-72-9; BOC-Gly-Ile-Val-γ-$C_6H_5CH_2O$-Glu-$NHNH_2$, 96573-69-2; BOC-Gly-Ile-Val-γ-$C_6H_5CH_2O$-Glu-N_3, 110434-08-7; insulin, 9004-10-8; S-sulfonated sheep insulin B-chain, 18175-60-5; [Asn^{21} diethylamide A]sheep insulin, 110485-99-9.

References

Arquilla, E. R., Bromer, W. W., & Mercola, D. A. (1969) *Diabetes 18*, 193–198.

Blundell, T. L., & Wood, S. P. (1975) *Nature (London) 257*, 197–203.

Blundell, T. L., Dodson, G., Hodgkin, D., & Mercola, D. (1972) *Adv. Protein Chem. 26*, 279–402.

Burke, G. T., Chanley, J. D., Okada, Y., Cosmatos, A., Ferderigos, N., & Katsoyannis, P. G. (1980) *Biochemistry 19*, 4547–4556.

Chu, Y.-C., Wang, R.-Y., Burke, G. T., Chanley, J. D., & Katsoyannis, P. G. (1987a) *Biochemistry* (first paper of three in this issue).

Chu, Y.-C., Wang, R.-Y., Burke, G. T., Chanley, J. D., & Katsoyannis, P. G. (1987b) *Biochemistry* (third paper of three in this issue).

Cosmatos, A., Cheng, K., Okada, Y., & Katsoyannis, P. G. (1978) *J. Biol. Chem. 253*, 6586–6590.

Coy, D. H., Vilchez-Martinez, J. A., Coy, E. J., Nishi, N., Arimura, A., & Schally, A. V. (1975) *Biochemistry 14*, 1848–1851.

Ferderigos, N., Burke, G. T., Kitagawa, K., & Katsoyannis, P. G. (1983) *J. Protein Chem. 2*, 147–170.

Fujino, M., Kobayashi, S., Obayashi, M., Shinagawa, S., Fukuda, T., Kitada, C., Nakayama, R., & Yamazaki, I. (1972) *Biochem. Biophys. Res. Commun. 49*, 863–869.
Katsoyannis, P. G., Trakatellis, A. C., Johnson, S., Zalut, C., & Schwartz, G. (1967a) *Biochemistry 6*, 2642–2655.
Katsoyannis, P. G., Trakatellis, A. G., Zalut, C., Johnson, S., Tometsko, A., Schwartz, G., & Ginos, J. (1967b) *Biochemistry 6*, 2656–2668.
Ogawa, H., Burke, G. T., & Katsoyannis, P. G. (1984) *J. Protein Chem. 3*, 327–348.
Pullen, R. A., Lindsay, D. G., Wood, S. P., Tickles, I. J., Blundell, T. L., Wollmer, A., Krail, G., Brandenberg, D., Zahn, H., Gliemann, J., & Gammeltoft, S. (1976) *Nature (London) 259*, 369–373.
Schwartz, G., Burke, G. T., Chanley, J. D., & Katsoyannis, P. G. (1983) *Biochemistry 22*, 4561–4567.

Possible Involvement of the A^{20}–A^{21} Peptide Bond in the Expression of the Biological Activity of Insulin. 3. [21-Desasparagine,20-cysteine ethylamide-A]insulin and [21-Desasparagine,20-cysteine 2,2,2-trifluoroethylamide-A]insulin†

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ABSTRACT: We have synthesized [21-desasparagine,20-cysteine ethylamide-A]insulin and [21-desasparagine,20-cysteine 2,2,2-trifluoroethylamide-A]insulin, which differ from natural insulin in that the C-terminal amino residue of the A chain, asparagine, has been removed and the resulting free carboxyl group of the A^{20} cysteine residue has been converted to an ethylamide and a trifluoroethylamide group, respectively. [21-Desasparagine,20-cysteine ethylamide-A]insulin displayed equivalent potency in receptor binding and biological activity, ca. 12% and ca. 14%, respectively, relative to bovine insulin. In contrast, [21-desasparagine,20-cysteine 2,2,2-trifluoroethylamide-A]insulin displayed a divergence in these properties, ca. 13% in receptor binding and ca. 6% in biological activity. This disparity is ascribed to a difference in the electronic state of the A^{20}–A^{21} amide bond in these two analogues. A model is proposed to account for the observation of divergence between receptor binding and biological activity in a number of synthetic insulin analogues and naturally occurring insulins. In this model, changes in the electronic state and/or the orientation of the A^{20}–A^{21} amide bond can modulate biological activity independently of receptor binding affinity. The A^{20}–A^{21} amide bond is thus considered as an important element in the "message region" of insulin.

In the previous two papers (Chu et al., 1987a,b), we have implicated A^{20}–A^{21} amide bond as a necessary structural feature for the expression of the biological activity of insulin and have suggested that this activity is modulated by the electronic state of the A^{20}–A^{21} amide bond. This may represent an important element of the "message region" of insulin. To further probe this speculation, we here describe the synthesis and biological evaluation of [21-desasparagine,20-cysteine ethylamide-A]insulin ([des-A^{21}Asn,A^{20}cysteine ethylamide]insulin) and [21-desasparagine,20-cysteine 2,2,2-trifluoroethylamide-A]insulin, ([des-A^{21}Asn,A^{20}cysteine trifluoroethylamide]insulin),[1] in which the C-terminal residue, asparagine, of the A chain has been eliminated and the resulting free carboxyl group at position A^{20} has been converted to an ethylamide and a trifluoroethylamide, respectively.

Experimental Procedures and Results

Details of materials, analytical procedures, and biological evaluation by lipogenesis assays in isolated rat fat cells, receptor binding assays in rat liver plasma membranes, and radioimmunoassays are given in a previous publication (Chu et al., 1987a). Receptor binding potency in isolated rat fat cells was examined as previously described (Burke et al., 1980).

General Aspects of Synthesis of [Des-A^{21}Asn,A^{20}cysteine ethylamide]- and [Des-A^{21}Asn,A^{20}cysteine trifluoroethylamide]insulins. The synthesis of these analogues was carried out by the interaction of the S-sulfonated bovine (sheep) B chain with the respective S-sulfonated A chain analogue as described previously (Chu et al., 1987a). The overall procedure for the preparation of the S-sulfonated A chain analogues is comparable to that outlined previously (Chu et al., 1987a).

*Glycyl-*L*-isoleucyl-*L*-valyl-*L*-glutamyl-*L*-glutaminyl-S-sulfo-*L*-cysteinyl-S-sulfo-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-S-sulfo-*L*-cysteinyl-*L*-seryl-*L*-leucyl-*L*-tyrosyl-*L*-glutaminyl-*L*-leucyl-*L*-glutamyl-*L*-asparaginyl-*L*-tyrosyl-S-sulfo-*L*-cysteine*

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[1] Abbreviations: AcOH, acetic acid; CM, carboxymethyl; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; HMPA, hexamethylphosphoramide; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; TEA, triethylamine; THF, tetrahydrofuran; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride. Compounds designated by Roman numerals are described fully in the supplementary material (see paragraph at end of paper regarding supplementary material). The IUPAC–IUB name of [21-desasparagine,20-cysteine ethylamide-A]insulin, for example, is des-A21-asparagine-[20-cysteine ethylamide]insulin (des-Asn^{A21}-[A20-cysteine ethylamide]insulin).

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