Tetrahedron Vol 39, No. 1, pp. 149 to 158, 1983
Printed in Great Britain.

0040-4020/83/010149-10$03.00/0
Pergamon Press Ltd.

THE SYNTHESIS OF AN INSULIN ACTIVE SITE ANALOGUE

I. J. GALPIN*, G. HANCOCK and G. W. KENNER†
The Robert Robinson Laboratories, The University of Liverpool, P. O. Box 147, Liverpool, L69 3BX, England

and

B. A. MORGAN‡
Reckitt and Colman Limited, Pharmaceutical Division, Dansom Lane, Hull, HU8 7OS, England

(*Received in the U.K. 11 January* 1982)

Abstract—An insulin active site analogue has been prepared which incorporates all but two of the amino-acids which are found at the proposed active site of insulin. The cyclic molecule containing portions of the A and B chains was held in the required conformation by a disulphide linkage. The analogue was unfortunately not significantly active when compared in two insulin assays.

The syntheses of insulin achieved by several research groups[1-4] have permitted detailed study of the structure and mode of action of Insulin. These syntheses prompted us to attempt the synthesis of an insulin analogue which would retain the essential features of the active site/receptor binding region in the hope that it would mimic the action of the parent hormone and thus bring about a response similar to that of insulin. It was hoped that by synthesising an insulin analogue of reduced size it would be possible to examine the receptor binding site and thus learn more about the mode of action of the hormone. Also, the synthesis of such a small analogue might eventually lead to a compound with insulin like activity which could be used therapeutically.

Many structure activity studies have been carried out in recent years and these have confirmed the presence of many invariant residues lying mainly on the surface of the insulin molecule. This region includes Al glycine, A4 glutamic acid, A5 glutamine, A19 tyrosine, A21 asparagine, B12 valine, B16 tyrosine, B22 arginine, B23 glycine, B24 phenylalanine, B25 phenylalanine and B26 tyrosine. Experiments on modified insulins by many different workers have shown that any chemical modification leading to an alteration in the tertiary structure results in a reduction in receptor binding, and it is therefore important to maintain the positions of the invariant residues found in the parent hormone when designing an active site analogue.

We therefore constructed an accurate molecular model of insulin based on the published X-ray structure;[5] this allowed us to select those topographical features which should be present in our active site analogue.

Examination of the model indicated that a large part of the structure required for activity could be correctly spatially placed by linking the A19–21 fragment to the B19–27 fragment through the natural A20/B19 disulphide linkage. Connection of the essential A(1–5) amino terminal region presented a more difficult problem as no natural linkages were available. From a close inspection of the model it was clear that the carboxyl terminal region of the B chain and the A chain (1–5) sequence were in close proximity, and that B27 threonine and A3 valine were within 6Å of one another. This prompted us to link these residues covalently by substituting a lysine residue for the non-critical A3 valine and forming an amide bond from the side chain of lysine to the carboxyl terminus of the B chain at alanine-27. We replaced threonine at B27 by alanine in an attempt to simplify the coupling at this point. Similarly, we found that the A2 isoleucine and A18 asparagine side-chains were in close proximity in the insulin molecule. Thus, replacement of isoleucine by lysine and replacement of asparagine with aspartic acid permitted covalent connection to be achieved through the epsilon amino function of the lysine and the carboxyl side-chain of aspartic acid. The slightly less critical residues B12 valine and B16 tyrosine were omitted from our analogue.

In order to maintain the electrical charge balance of the parent molecule the alpha amino functions of A18 aspartic acid and B19 cysteine were deleted giving succinic acid and β-thiopropionic acid respectively. Also, the A5 glutamine carboxyl was amidated. The resulting analogue structure (Fig. 1) clearly contained several major modifications to the normal structure of the active-site. However, it was hoped that the precise positioning

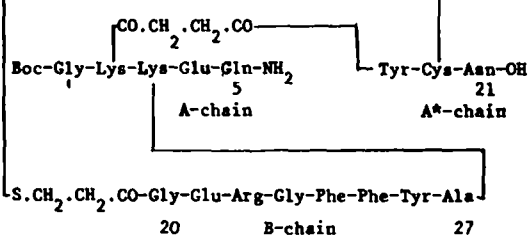


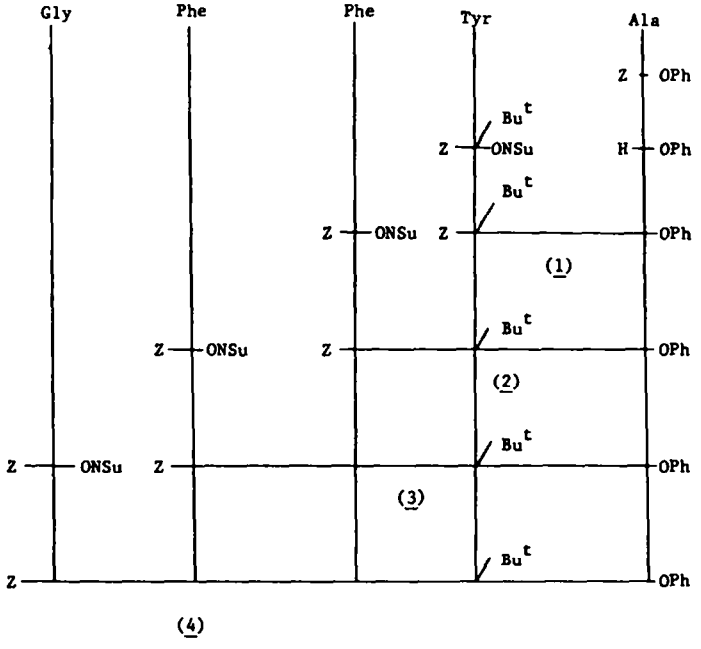
Fig. 1. Insulin active site analogue (30).

of invariant residues which was permitted by the introduction of such unusual connecting linkages would result in the analogue having a three dimensional structure in which the residues contained in the analogue were in very similar positions to those found in the natural hormone. It is also worth making the point that such unnatural linkages should render the analogue more stable to enzymes than the natural peptide. For synthetic convenience the analogue was broken into three major fragments, the A 1–5, A* 19–21 and B chain fragments.

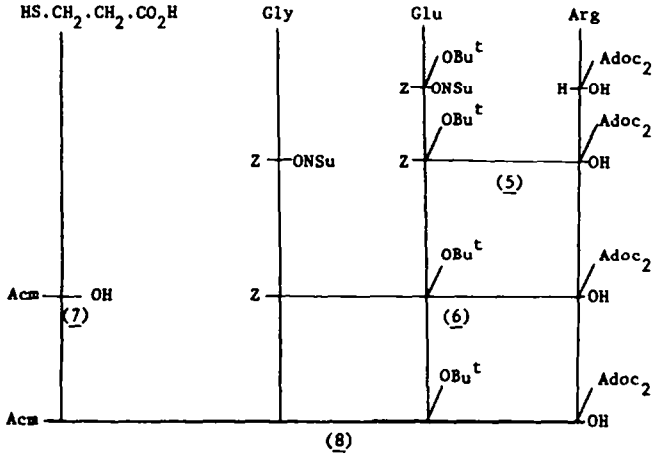
†Deceased 25th June 1978.
‡Present address: Sterling-Winthrop Research Institute, Columbia Turnpike, Rensselaer, NY 12144, USA.

During the synthesis we used well established protecting groups[6] whenever possible, thus benzyloxycarbonyl was used for temporary protection of alpha amino functions, unless sulphur was present, in which case t-butyloxycarbonyl was used instead. Phenyl[7] and t-butyl esters were used for carboxyl protection where appropriate and t-butyl ethers and esters were used for protection of side chain hydroxyl and carboxyl functions. The bis(adamantyloxycarbonyl) derivative of arginine[8] was used in this synthesis. Protection of thiols was achieved using the acetamidomethyl group which was retained until the very last stage in the synthesis. The selective protection of lysine at residues two and three in the A chain was achieved by protecting lysine-3 as its trichloroethoxycarbonyl derivative,[9,10] as this should be stable to hydrogenation over palladium on charcoal during fragment extension. The epsilon amino function of lysine-2 was initially blocked as the half phenyl furmarate, reduction finally giving a succinyl linkage.

In order to facilitate the synthesis of the B chain it was divided at arginine-22 into two approximately equal portions. The (23–27) penta peptide was assembled by the route shown in Scheme 1. The protected fragment was assembled by stepwise extension using hydroxysuccinimide activated esters. Intermediate removal of the benzyloxycarbonyl group being achieved in the usual way by catalytic hydrogenation in the presence of toluene p-sulphonic acid. Although we were slightly apprehensive about the hydrogenation of the dipeptide 1 we found that extension to the tripeptide 2 was achieved in 85% yield with no signs of any diketopiperazine formation. Following the addition of a second phenylalanine residue, the tetrapeptide 3 was extended to the fully protected pentapeptide 4, this was adequately purified by recrystallization from THF/water. The fragment thus produced was used directly in the preparation of the required B chain sequence. The synthesis of the remaining B chain fragment was carried out according to Scheme 2. The bis(adamantyloxycarbonyl) arginine was



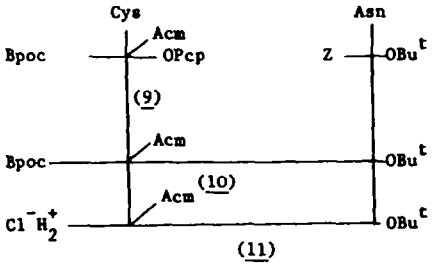
Scheme 1. Synthesis of the B-chain 23–27 fragment.



Scheme 2. Synthesis of modified B-chain 20–22

obtained by hydrogenolysis of the corresponding benzyloxycarbonyl derivative by the method of Geiger[8] including slight modifications as we have indicated previously.[11] Salt coupling of the arginine derivative employing the triethylammonium salt and Z.Glu(OBu^t).ONSu[12] gave the dipeptide **5**. Extension to the tripeptide **6** was readily achieved again using the active ester procedure used in previous couplings. *S*-Acetamidomethyl β-thiopropionic acid **7**[13] was obtained as an oil from the reaction of β-thiopropionic acid and hydroxymethyl acetamide in trifluoroacetic acid at room temperature. This acid was then coupled with the tripeptide **6** after hydrogenolysis of the benzyloxycarbonyl group, on this occasion activation of the carboxyl component was achieved with pivaloyl chloride, the resulting product **8** being obtained in 59% yield.

The Cys-Asn dipeptide used in the preparation of the A* fragment was synthesised according to Scheme 3.



Scheme 3. Synthesis of A* fragment

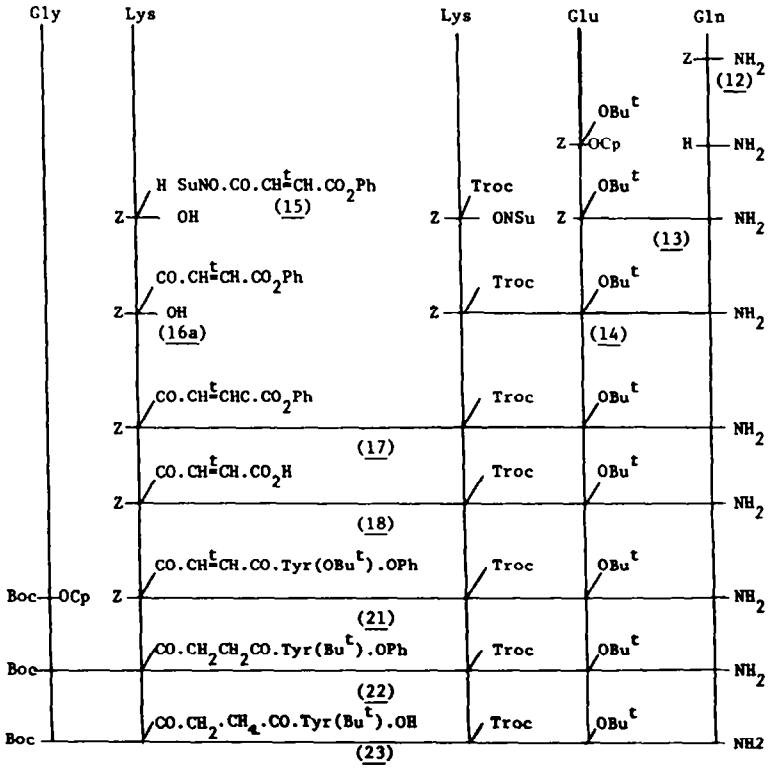
The Z.Asn.OBu^t[14] was straightforwardly prepared by the literature method by the reaction of benzyloxycarbonyl asparagine with isobutylene in the presence of concentrated sulphuric acid. The active ester of cysteine **9** was prepared by reaction of Bpoc.Cys(Acm).OH[15] with pentafluorophenol in the presence of DCCI. Reaction of this active ester with the appropriate amino component obtained by hydrogenolysis gave the fully protected dipeptide **10**. Unfortunately we had to resort to gel filtration purification using Sephadex LH20 eluting with DMF in order to achieve purification, and even then a yield of only 35% was recorded. Alternative methods of coupling gave less satisfactory results. Removal of the amino protecting group from **10** using the 0.1M HCl in 90% trifluoroethanol-water at a pH of 0.5[16] gave the required hydrochloride **11** after 30 min.

The main A-chain portion was synthesized according to the Scheme 4. Assembly of the tri-peptide **14** was achieved using an active ester route through compound **12** and **13**. The glutamyl residue was introduced as its active trichlophenyl ester with the addition of hydroxybenzotrizole being used to increase the yield and shorten the reaction time. The lysyl residue was also introduced by an active ester route, on this occasion employing hydroxysuccinamide as the activating ester.

The lysine derivative **16a** required for chain extension was synthesised from Z.Lys.OH.[17] This derivative being reacted with the N-hydroxysuccinamide ester of phenyl fumerate **15**.[18] The reaction, which was carried out in 15% aqueous DMF, gave the required product **16** but in order to facilitate isolation and purification, this acid was converted to the corresponding dicyclohexylammonium salt.

It was found to be impossible to use the succinyl linkage at this stage as the side chain amide nitrogen cyclised to displace phenoxide from the succinyl phenyl ester giving a cyclic product (Fig. 2).

Such a reaction was not observed in the case of the half phenyl fumerate **16a**; however, as a precaution we



Scheme 4. Synthesis of A-chain fragment.

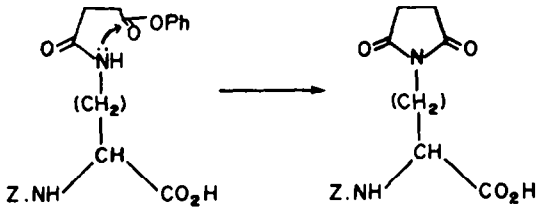


Fig. 2. Cyclisation of succinyl side-chain.

checked that the phenyl ester could be cleaved under standard condition[7] leaving the olefinic double bond intact.

In order to couple the modified lysine residue **16a** it was necessary to remove the benzyloxycarbonyl group from tripeptide **14**. Overnight catalytic hydrogenolysis in the presence of 10% palladium on charcoal and p-toluene sulphonic acid followed by DCCI/HOBt coupling of **16a** gave a yield of only 26% after chromatography on Sephadex LH20. A detailed investigation soon indicated that the trichloethoxycarbonyl protecting group was unstable to catalytic hydrogenolysis.[19] This instability is contrary to the findings of the workers who originally developed the group.[9,10]

After optimising the condition of cleavage, we found that 5% palladium on charcoal was a more suitable catalyst and that hydrogenenolysis could not be continued for longer than 8 hr. Using this procedure for benzyloxycarbonyl cleavage and coupling by the DCCI/HOBt method, we were eventually able to obtain the required modified tetrapeptide **17** after gel filtration on Sephadex LH20 eluting with DMF.

The phenyl ester group was then cleaved from the phenyl fumarate side-chain of **17** under our standard conditions[7] using one equivalent of hydrogen peroxide at pH. 10.5. The resulting acid **18** was then coupled to the toluene p-sulphonate salt of H.Tyr(Bu^t).OPh **20**. This salt was produced from the corresponding benzyloxycarbonyl derivative **19** by hydrogenolysis under standard conditions. The compound **19** being obtained from Z. Tyr(Bu^t).OH[20] by reaction with DCCI and phenol. The coupled product **21** was obtained in 82% yield after reprecipitation from DMF/water.

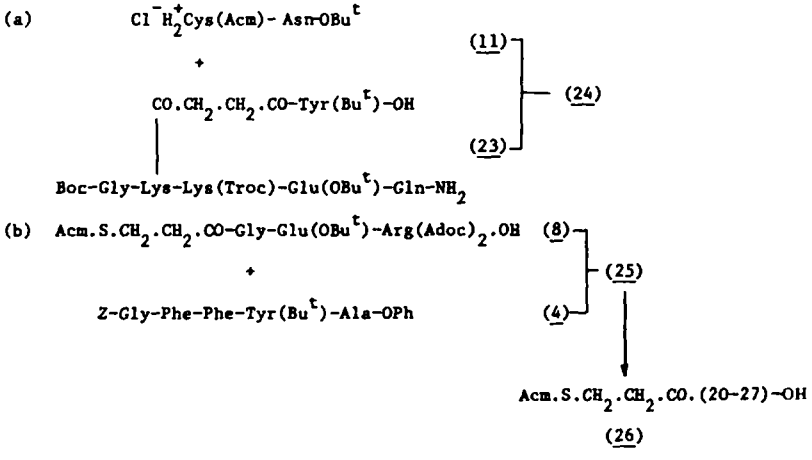
The protected peptide **21** was then hydrogenolysed in the presence of 5% palladium charcoal for eight hours. The resulting salt was coupled with Boc.Gly.OCp[21] giving the fully protected product **22**. It should be noted that the reductive conditions employed for the removal of the benzyloxycarbonyl group converted the olefinic fumaryl linkage to the corresponding succinyl linkage. These conditions did not affect the troc group adversely, as in this case a 64% yield of the corresponding toluene-p sulphonate salt of **21** was obtained.

The tyrosyl phenyl ester in the compound **22** was then cleaved under the standard conditions[7] to give the corresponding acid **23** which was used directly in the subsequent coupling reaction to form the bridged A–A* fragment.

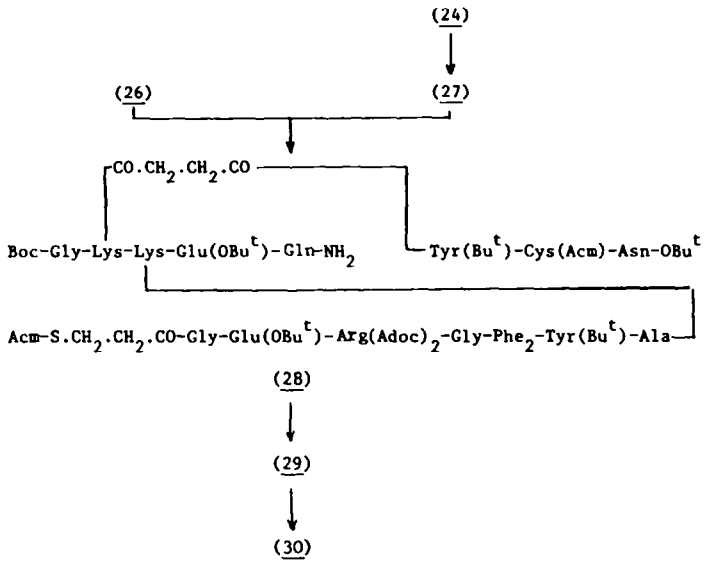
The combination of the A and A* sub-fragments was then carried out by the route shown in Scheme 5(a). The peptide acid **23** and the hydrochloride **11** were combined using a pre-activation technique with DCCI and HOBt. A coupling ratio of 1 : 1.5 was used in order to maximise the yield. In any event the coupled product **24** could only be obtained in 42% yield after chromatography on silica gel. The B chain sub-fragments were combined as indicated in Scheme 5(b). The protected peptide **25** being obtained in 59% yield after recrystallisation. This peptide phenyl ester was then subjected to standard phenyl ester cleavage conditions[7] to yield the peptide acid **26**.

The trichloroethoxycarbonyl group had then to be selectedly removed from the second lysine residue in the A chain fragment **24**. Using the published procedure[9,10] employing zinc in glacial acetic acid, two products were produced which were very difficult to separate. In order to get around this problem we employed a variety of conditions with zinc as the cleavage agent. In all cases at least two products were observed. The close similarity between zinc and cadmium then prompted us to attempt the use of cadmium in the cleavage reaction.[19] Immediately it was clear that the troc protection on the second lysine of the octapeptide **24** was readily removed on treatment with cadmium dust in a 1 : 1 mixture of acetic acid/DMF. Overnight reaction gave a single product which was purified using gel filtration, employing Sephadex LH20 eluting with DMF. Ultimately the hydrochloride of this peptide was obtained in 85% overall yield. The compound was shown to be homogeneous by tlc and to have a good amino acid analysis, 220 MHz proton NMR indicated that the troc group had been completely removed and that the other protecting groups had remained intact during this cleavage.

The A and B chains were then linked using the DCCI/HOBt procedure as indicated in Scheme 6. The



Scheme 5. Combination of A and B-chain sub-fragments.



Scheme 6. Assembly of the insulin active-site analogue.

amide linkage was formed between the terminal alanine of the B chain and lysine-3 of the A chain. The fully protected link compound **28** was obtained after gel filtration on Sephadex LH60 eluting with DMF. Unfortunately, only a 30% yield of the coupled product **28** was obtained, but this was fully homogeneous by tlc and had a good amino acid analysis and combustion analysis. The 360 MHz proton Nmr clearly showed the presence of A and B chain residues and the protecting groups on each residue in the appropriate chain. At this stage with d_6-DMSO as solvent, we were able to see the Acm CH_3 and Acm CH_2 groups in both the A and the B chains separately.

The fully protected material **28** was then deprotected by dissolving in 90% trifluoroacetic acid in the presence of ethane-1-2-dithiol. After four hours the product **29** was purified by gel filtration on carboxymethyl cellulose, final purification being achieved by desalting on a Sephadex G15 column eluting with 50% acetic acid. A yield of 48% was recorded for the compound **29** which was shown to be homogeneous by tlc and by electrophoresis. Amino acid analysis and 360 MHz proton Nmr served to authenticate the integrity of the compound. Unfortunately, at this stage d_4-acetic acid had to be used as the solvent for proton Nmr and it was found that the Acm CH_3 signal was obscured by residual protons in the solvent. However, we were clearly able to see the signals arising from the Acm CH_2 groups on both the A and B chains.

In the final oxidation step to produce the insulin analogue **30** we followed as closely as possible the conditions employed by the Ciba Geigy group in their synthesis of human insulin.[4] The Acm protecting groups were removed with the simultaneous formation of disulphide by treatment with iodine at high dilution in acetic acid. Initially, we used ascorbic acid for decolourisation at the end of the reaction, but finally we chose sodium thiosulphate as the decolourising agent, as this was more readily removed during the work up. The final product **30** was initially purified by gel filtration on Sephadex G15 eluting with 50% acetic acid. The major peak eluting with Ve/Vt ratio 0.50 was further purified by gradient elution on carboxymethyl cellulose. Finally, the product was desalted by gel filtration on Sephadex G15 eluting with 50% acetic acid. The final product being isolated in an overall yield of 40%. The product was homogeneous by tlc and by electrophoresis at pH 1.9. The amino acid analysis was satisfactory, although a slightly high value for lysine was recorded. A modified programme on the amino acid analyser showed that no ornithine had been formed from arginine during the preparation of the compound as we suspected this as a possible explanation for the low arginine and high lysine/ornithine figure from other work in our group. On this occasion the 360 MHz proton NMR spectrum in d_4-HOAc showed the absence of any peaks attributable to the Acm protecting group, all other residues gave their anticipated signals. A portion of the 360 MHz proton Nmr spectrum is shown in Fig. 3 and from this it can be seen that the Acm group has been completely removed by the absence of the peak due to the CH_2 protons in the spectrum of compound **30**. A similar spectrum of the Acm containing unoxidised material **29** clearly shows a singlet at this position, due to the CH_2 group of Acm. The change in optical rotation which was observed on oxidation of the compound **29** also suggested that satisfactory oxidation to the compound **30** had been achieved.

An investigation into the receptor binding characteristics and *in vitro* activity of the analogue **30** was carried out. The results showed that the analogue **30** in a concentration of 10 μg/ml does not stimulate glucose oxidation above basal. Also, that it exhibits very little competition with I^{125} monoiodoinsulin for the receptors of rat adipoocytes. Control experiments showed a clear cut effect should have been observed at this level if the analogue **30** was significantly active, but it was calculated that the peptide had a biological potency of probably less than 0.001% of that of natural insulin. It is worth noting that our analogue **30** and an insulin analogue produced by the Ciba-Geigy group[22] with a related skeleton, both showed very little biological activity. Thus, the simplification involved in the synthesis must have resulted in the omission of certain essential residues which were more important to binding and activity than had appeared from the results on synthetic insulins.

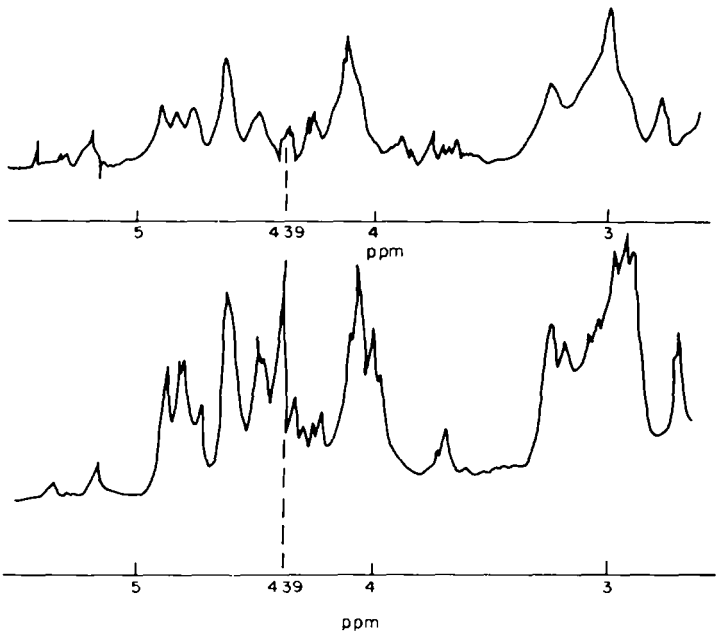


Fig. 3. Portion of the H NMR spectrum (360 MHz) of compounds (**29** and **30** showing loss of signal due to ACM-CH_2.

EXPERIMENTAL

Abbreviations not in common usage are as follows: DCCI dicyclohexylcarbodiimide; DCHA, dicyclohexylammonium; DMF, dimethylformamide; DMS, dimethylsulphide; HOBt, N-hydroxybenzotriazole; HONSu, *N*-hydroxysuccinimide; IPA, iso-propanol; NMM, *N*-methylmorpholine; TEA, triethylamine; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; THF, tetrahydrofuran. Tlc was carried out on silicagel GF_{254} using Merck prepared plates in the following systems: (1) $CHCl_3$/IPA 3/1; (2) as (1), but 7/1; (3) as (1) but 9/1; (4) $CHCl_3$/MeOH 9/1; (5) EtOAc/IPA/HOAc/H_2O 240/20/6/11; (6) as (5) but 120/20/6/11; (7) EtOAc/Pyridine/HOAc/H_2O 240/20/6/11; (8) $CHCl_3$/MeOH/AcOH 8/1/1; (9) as (7) but 120/20/6/11; (10) as (7) but 60/20/6/11; (11) ^{n}BuOH/HOAc/H_2O 3/1/1; (12) as (4) but 5/1; (13) as (5) but 60/20/6/11; (14) $CHCl_3$/MeOH/HOAc/H_2O 60/18/2/3; (15) as (4) but 80/20/2/3; (16) BuOH/pyridine/HOAc/H_2O 60/20/6/24; (17) as (7) but 5/5/1/3; (18) BuOH/pyridine/$H.CO_2H$/H_2O 44/24/2/20. Compounds were visualised by one or more of the following methods: iodine vapour, chlorine/starch/KI, ninhydrin, fluorescence and UV absorption at 254 nm. Free peptides were subject to electrophoresis at pH 1.9, E^{19} being the mobility relative to ε-Dnp.Lys.HCl. Amino-acid analysis was carried out on a Jeol 6AH after sealed tube hydrolysis with 6M HCl at 110° for 24h.

Scheme 1

Z-Tyr(Bu^t)-Ala-OPh(1) A solution of Z-Tyr(Bu^t)-ONSu[23] (10.3 g, 22 mM), Tos $O^-H_2^+$-Ala-OPh[7] (6.7 g, 20 mM) and NMM (2.0 g, 2 mM) in DMF (50 ml) was stirred at room temperature overnight. The solvent was evaporated *in vacuo* and the residue dissolved in EtOAc and washed with dilute acid and base in the usual way. After drying, the EtOAc was evaporated to give a white solid which was recrystallised from IPA/H_2O to give **1** (7.3 g, 70%), m.p. 134–136°, $\{\alpha\}_D^{22} - 32.4°$ (c = 1, DMF, $R_f(1) - 0.85$, $R_f(2) - 0.8$, $Ala_{1.01}Tyr_{0.99}$ (Found: C, 69.53; H, 6.60: N, 5.37. $C_{30}H_{34}N_2O_6$ requires: C, 69.48: H, 6.61; N, 5.40%).

Z-Phe-Tyr(But)-Ala-OPh **2**: Compound **1** (8.3 g, 16 mM) and Tos.OH.H_2O (3.9g, 16 mM) were dissolved in DMF (70 ml) and hydrogenolysed for 18h over 10% Pd/C (0.8 g). Filtration and evaporation gave the salt after trituration with EtOAc/petroleum ether (60–80°) as a white solid (6.5 g, 73%). This material was dissolved in DMF (20 ml) and cooled to 0° prior to the addition of Z-Phe-ONSu[24] (3.9 g, 9.9 mM) and NMM (0.91 g, 9 mM). The reaction mixture was stirred overnight then evaporated and dissolved in EtOAc. After washing with acid and base the solution was dried and evaporated to give a residue which was crystallised from MeOH to give **2** (5.0 g, 83%), m.p. 178–180°, $[\alpha]_D^{22} - 44.2°$ (c = 1, DMF), $R_f(1) - 0.9$, $R_f(3) - 0.7$, $Ala_{1.04}Tyr_{0.97}Phe_{1.00}$ (Found: C, 70.17; H, 6.33; N, 6.41. $C_{39}H_{43}N_3O_7$ requires: C, 70.36; H, 6.51; N, 6.31%).

Z-Phe-Phe-Tyr(Bu^t)-Ala-OPh **3**. The protected tripeptide **2** (5.0 g, 7.5 mM) was hydrogenolysed in the presence of Tos.OH.H_2O (1.4 g, 7.5 mM) and 10% Pd/C (50 mg) then worked up in the usual way. The resulting salt was coupled with Z-Phe-ONSu[24] (2.7 g, 6.7 mM) in the presence of NMM (0.6 g, 6.1 mM) as in the previous experiment using DMF (20 ml) as solvent. Evaporation gave a residue which was triturated with 10% citric acid solution, the resulting solid being recrystallised from THF/H_2O to give the required product **3** (3.1 g, 63%), m.p. 218–222°, $[\alpha]_D^{22} - 40.7°$ (c = 1, DMF), $R_f(2) - 0.8$, $R_f(4) - 0.8$, $Ala_{1.02}Tyr_{0.99}Phe_{1.99}$, (Found: C, 70.65; H, 6.32; N, 7.08. $C_{48}H_{43}N_4O_8$ requires: C, 70.92; H, 6.45; N, 6.89%).

Z-Gly-Phe-Phe-Tyr(Bu^t)-Ala-OPh **4**. The compound **3** (3.1 g, 3.8 mM) and Tos.OH.H_2O (0.7 g, 3.8 mM) were dissolved in DMF (70 ml) and hydrogenolysed over 10%Pd/C (0.3 g) overnight. Filtration and evaporation gave a white solid which was dissolved in DMF (7 ml). After cooling to 0° Z-Gly-ONSu[24] (1.1 g, 3.9 mM) and NMM (0.36 g, 3.5 mM) were added and the reaction mixture stirred overnight at room temperature. The solvent was evaporated *in vacuo* and the residue triturated with 10% citric acid solution to produce a white solid product. This was recrystallised from THF/H_2O to yield **4** (2.5 g, 82%), m.p. 210–214°$\{\alpha\}_D^{22} - 26.7°$, (c = 1, DMF), $R_f(2) - 0.7$, $R_f(5) - 0.9$, $Gly_{0.96}Ala_{0.97}Tyr_{1.03}Phe_{2.03}$ (Found: C, 68.36; H, 6.38: N, 8.11. $C_{50}H_{55}N_5O_9 \cdot 0.5\ H_2O$ requires: C, 68.32; H, 6.42; N, 7.90%).

Scheme 2

Z-Glu(OBu^t)-Arg(Adoc)$_2$-OH **5**. H-Arg(Adoc)$_2$-OH[5] (2.8 g, 5 mM) and TEA (0.51 g, 5 mM) were dissolved in a mixture of water (18 ml) and DMF (2 ml). The solution was cooled to 0° and Z-Glu(Bu^t)-ONSu (2.4 g, 5.5 mM) added. The solution was allowed to warm to room temperature overnight then evaporated *in vacuo* to give a residue which was dissolved in EtOAc and washed with 10% ice cold citric acid. Drying and evaporation of the organic phase gave a clear oil which was crystallised from MeOH/H_2O giving the product **5** (3.5 g, 82%), m.p. 122–124°,

$\{\alpha\}_D^{22} - 3.8°$ (c = 1, DMF), $R_f(3) - 0.5$, $R_f(4) - 0.4$, $Arg_{0.95}$ $Glu_{1.05}$ (Found: C, 62.20; H, 7.52: N, 8.28. $C_{45}H_{63}N_5O_{11}$. H_2O requires: C, 62.27; H, 7.55; N, 8.07%).

Z-Gly-Glu(OBu^t)-Arg(Adoc)$_2$-OH **6**. The protected dipeptide acid **5** (2.6 g, 3 mM and Tos.OH.H_2O (0.57 g, 3 mM) were dissolved in DMF (25 ml) and hydrogenolysed overnight in the presence of 10% Pd/C (0.26 g). Work up in the usual way gave the salt as a white solid after trituration with water. After drying over P_2O_5 the salt (1.8 g, 2 mM) and TEA (0.4 g, 4 mM) were dissolved in a mixture of H_2O and DMF (6 ml/1 ml) and the solution cooled to 0°. Z-GlyONSu[24] (0.64 g, 2.2 mM) was added and the reaction mixture allowed to reach room temperature overnight. The solvent was removed *in vacuo* and the residue triturated with 10% citric acid and water to yield a white solid which was recrystallised from MeOH/H_2O giving **6** (1.3 g, 70%), m.p. 110–114° (dec), $\{\alpha\}_D^{22} - 6.7°$ (c = 1, DMF), $R_f(4) - 0.5$, $R_f(6) - 0.6$, $Arg_{0.99}$ $Glu_{1.03}$ $Gly_{0.98}$ (Found: C, 60.32; H, 7.22; N, 9.19. $C_{47}H_{66}N_6O_{12}$. 1.5 H_2O requires: C, 60.43; H, 7.44; N, 9.00%).

Acm-S.CH_2.CO_2H **7**[8]. 3-Mercaptopropionic acid (7.4 g, 0.1 mM) and Acm.OH (8.9 g, 0.1 mM) were dissolved in anhydrous TFA (100 ml) and stirred for 30 min at room temperature. The solvent was evaporated to yield a pale yellow oil which could not be crystallised (9.0 g, 62%), $R_f(1) - 0.2$, $R_f(6) - 0.6$, NMR (60 MHz, d_6-DMSO) δ(ppm): 1.85(s, 3H CH_3.CO.), 2.68 (m, 4H, .CH_2.CH_2.), 4.29 (d, 2H, NH.CH_2.S) 8.48 (t, 1H CO.NH.CH_2), 12.45 (s, H, CO_2H).

Acm-S.CH_2.CH_2CO.Gly.Glu (OBu^t).Arg(Adoc)$_2$.OH **8**. The compound **6** (1.3 g, 1.5 mM) and Tos.OH.H_2O (0.29 g, 1.5 mM) were dissolved in DMF (25 ml) and hydrogenolysed overnight in the presence of 10% Pd/C (0.14 g). Work up in the usual way gave the salt as a white solid which was used directly in the coupling reaction.

Compound **7** (0.46 g, 2.6 mM) and NMM (0.29 ml), 2.6 mM) were dissolved in DMF (10 ml) and cooled to −15°. Pivaloyl chloride (0.31 g, 2.6 mM) was added and 30 min. allowed for activation prior to the addition of the toluene p-sulphonate from the hydrogenolysis and NMM (0.29 ml, 2.6 mM) in DMF (15 ml). After 2h the solvent was evaporated and the residue triturated with water. The resulting solid was triturated further with water, 10% citric acid and Et_2O, giving the required product **8** (0.74 g, 59%), m.p. 136 – 140° (dec), $\{\alpha\}_D^{22} - 7.2°$ (c = 0.5, DMF), $R_f(4) - 0.1$, $R_f(5) - 0.7$, $Arg_{1.00}Glu_{0.99}Gly_{1.00}$ (Found: C, 56.96; H, 7.50; N, 10.14. $C_{45}H_{69}N_7O_{12}S.H_2O$ requires: C, 56.88; H, 7.53: N, 10.32%).

Scheme 3

Bpoc-Cys(Acm)-OPcp **9**. Bpoc-Cys(Acm)-OH[15] (4.3 g, 10 mM) and pentachlorophenol (2.9 g, 11 mM) were dissolved in dimethoxyethane (50 ml) and the solution cooled to 0°. DCCI (2.3 g, 11 mM) was added and the reaction mixtured allowed to warm to room temperature overnight. The resulting urea was removed by filtration (and the solvent evaporated *in vacuo* to give a residue which was crystallised from EtOAc/petroleum ether 60–80° giving the active ester **9** (5.1 g, 75%), m.p. 111–114°, $\{\alpha\}_D^{22} - 58.5°$ (c = 1, DMF), $R_f(1) - 0.6$, $R_f(4)$ (Found: C, 49.57; H, 3.81; N, 4.10; S, 4.97. $C_{28}H_{25}N_2O_5Cl_5S$ requires: C, 49.54; H, 3.71; N, 4.13; S, 4.72%).

Bpoc-Cys(Acm)-Asn-OBu^t **10**. Z-Asn-OBu^t[14] (2.6 g, 8 mM) and Tos.OH.H_2O (1.5 g, 8 mM) were dissolved in DMF (50 ml) and hydrogenolysed for 18h in the presence of 10% Pd/C (0.26 g). The catalyst was removed by filtration and the solvent evaporated to yield the salt as a white solid after trituration with Et_2O (2.8 g, 97%). The active ester **9** (3.0 g, 4.4 mM) and the above salt (1.4 g, 4 mM) were dissolved in DMF (20 ml) at 0° along with NMM (0.44 ml, 4 mM) and the reaction mixture allowed to warm to room temperature overnight. The solvent was removed and the residue dissolved in EtOAc prior to washing with acid and base. The solution was then dried and evaporated to give a white residue which was further purified by gel filtration on Sephadex LH20 eluting with DMF. The appropriate fractions were pooled (Ve/Vt − 0.48) and evaporated *in vacuo* to give the white solid product **10** (0.84 g, 35%), m.p. 100° (dec), $\{\alpha\}_D^{25} - 26.7°$ (c = 1, DMF), $R_f(7) - 0.4$, $R_f(8) - 0.8$, (Found: C, 57.85; H, 6.67; N, 8.66. $C_{30}H_{40}N_4SO_7$. 1.25H_2O requires C, 57.80; H, 6.87; N, 8.99%).

$Cl^-H_2^+$-Cys(Acm)-Asn-OBu^t **11**. The protected dipeptide **10** (0.6 g, 1 mM) was dissolved in a mixture of TFE (9 ml) and water (1 ml). DMS (3.6 ml, 50 mM) was added and the pH brought to 0.5 with 0.1 M HCl in 90% aqueous TFE. After 30 min. at this pH the cleavage was complete and the solvent was evaporated to give a residue which was triturated with Et_2O giving the required salt **11** (174 mg, 44%), m.p. 110 – 114°, $\{\alpha\}_D^{23} - 33.2°$ (c = 1, DMF), $R_f(9) - 0.2$, $R_f(10) - 0.2$ (Found: C, 39.67; H, 6.97; N, 13.20. $C_{14}H_{27}N_4SO_5Cl$.1.5 H_2O requires:C, 39.48; H, 7.10; N, 13.15%).

Scheme 4

Z-Gln-NH_2 **12**. Thionyl chloride (23.8 g, 200 mM) was added dropwise to MeOH (500 ml) at 0°, Z-Glu-OH[25] (28.1 g, 100 mM) was then added and the reaction mixture allowed to warm to room temperature over 6h. The solution was concentrated to a thick oil which was dissolved in MeOH (250 ml) and NH_3 bubbled through this solution at −10°. After 30 min the flask was stoppered and the solution left to stand at room temperature for two days. The solvent was evaporated to give a residue which was recrystallised from MeOH/Et_2O giving **12** (28.1 g, 50%) m.p. 195 – 198°, $\{\alpha\}_D^{22} + 8.5°$(c = 1, DMF), $R_f(7) - 0.7$, $R_f(10) - 0.4$, (Found: C, 55.94; H, 6.18; N, 15.24. $C_{17}H_{17}N_3O_4$ requires: C, 55.91; H, 6.13; N, 15.05%).

Z-Glu(OBu^t)-Gln-NH_2 **13**. Compound **12** (11.2 g, 40 mM) and Tos.OH.H_2O (7.6 g, 40 mM) were dissolved in DMF (100 ml) and hydrogenolysed for 18 h in the presence of 10% Pd/C (1.12 g). Work up in the usual way gave the salt as a white solid which was dissolved in DMF (100 ml). Z-Glu(OBu^t)-OCp[26] (18.6 g, 36 mM) and NMM (3.32 g, 33 mM) were then added after cooling to 0° followed by HOBt (4.4 g, 33 mM). After warming to room temperature overnight the solvent was removed and the residue triturated with 10% citric acid giving a white solid. After filtration, this material was recrystallised from MeOH giving **13** (11.8 g, 77%), m.p. 197–203° $\{\alpha\}_D^{22} - 1.9°$ (c = 1, DMF), $R_f(7) - 0.5$, $R_f(6) - 0.6$ (Found: C, 56.61; H, 6.83; N, 11.92. $C_{22}H_{32}N_4O_7$ requires: C, 56.89; H, 6.94; N, 12.06%).

Z-Lys(Troc)-Glu(OBu^t)-Gln-NH_2 **14**. The protected dipeptide **13** (11.6 g, 25 mM) and Tos.OH.H_2O (4.7 g, 25 mM) were dissolved in DMF (125 ml). After the addition of 10% Pd/C (1.16 g) hydrogen was passed through the suspension for 18 h. Filtration and evaporation gave the salt as a pale yellow oil which was used directly in the subsequent coupling reaction. Z-Lys(Troc).OH[27] (8.8 g, 20 mM) and HONSu (2.3 g, 20 mM) were dissolved in CH_3CN (75 ml) and the resulting solution cooled to 0°. DCCI (4.3 g, 21 mM) was then added and the reaction mixture warmed to room temperature overnight. After cooling and filtration the solvent was evaporated to yield the active ester as a pale yellow oil. This oil and the toluene p-sulphonate salt obtained above were dissolved in DMF (50 ml) at 0° along with NMM (2.0 g, 20 mM). The reaction mixture was allowed to reach room temperature overnight. The solvent was then evaporated and the residue triturated with 10% citric acid to give a white solid which was recrystallised from MeOH/Et_2O giving **14** (10.0 g, 65%), m.p. 194 – 198°, $\{\alpha\}_D^{23} - 13.6°$ (c = 0.5, DMF), $R_f(6) - 0.6$, $R_f(11) - 0.7$, $Lys_{0.98}Glu_{2.03}$ (Found: C, 48.41; H, 5.82; N, 10.82; Cl, 13.92. $C_{31}H_{45}N_6O_{10}Cl_3$ requires: C, 48.48; H, 5.91: N, 10.92; Cl, 13.85%).

Trans-PhO.CO.CH = CH.CO.ONSu **15**. Phenyl hydrogen fumarate[18] (9.6 g, 50 mM) and HONSu (6.0 g, 52.5 mM) were dissolved in CH_3CN and cooled to 0°. DCCI (10.9 g, 52.5 mM) was added and the reaction mixture allowed to warm to room temperature overnight. The resulting urea was removed by filtration and the solvent evaporated to give a residue which solidified on trituration with Et_2O. Recrystallisation from IPA gave **15** (7.5 g, 52%), m.p. 119–122° (Found: C, 58.01; H, 3.87; N, 5.07. $C_{14}H_{11}NO_6$ requires: C, 58.14; H, 3.83: N, 4.84%).

Z-Lys (trans-CO.CH=CH-CO_2Ph)OH.DCHA **16**. Z-Lys.OH[17] (3.7 g, 13 mM) and TEA (1.3 g, 13 mM) were dissolved in a mixture of water (4 ml) and DMF (22 ml). After cooling to 0° the active ester **15** (3.8 g, 13 mM) was added and stirring continued overnight at room temperature. The solvent was evaporated to yield an oil which was dissolved in EtOAc. This solution was washed with 10% citric acid and water, then dried and evaporated. The resulting oil was dissolved in EtOAc (30 ml) and dicyclohexylamine (2.4 g, 13 mM) added, filtration after stirring

overnight gave the crude salt which was recrystallised from IPA/petroleum ether, 60–80° to give **16** (5.0 g, 60%) m.p. 126–132°, $\{\alpha\}_D^{23}$ – 5.6°, (c = 1, DMF), $R_f(2)$ – 0.1, $R_f(11)$ – 0.7 (Found: C, 66.86; H, 7.74; N, 6.70. $C_{36}H_{49}N_3O_7$. 0.5 H_2O requires: C, 67.06; H, 7.82; N, 6.51%).

Z-Lys (trans-CO.CH=CH.CO_2Ph)-Lys (Troc)-Glu (OBu^t)-Gln-NH_2 **17**. Compound **14** (4.6 g, 6 mM) and Tos.OH.H_2O (1.1 g, 6 mM) were dissolved in DMF (60 ml) and after the addition of 5% Pd/C (0.46 g) hydrogen was passed through the solution for 8 h. Work up in the usual manner gave the salt as a white solid which was used directly in the coupling reaction. The salt **16** was converted to the corresponding acid (3.0, 6.6 mM) by the standard procedure using 10% citric acid. The liberated acid and HOBt (1.6 g, 12 mM) were dissolved in DMF (14 ml) and cooled to 0°. DCCI (1.4 g, 6.6 mM) was then added and the reaction mixture stirred for 30 min at 0°. The toluene p-sulphonate obtained above was then added along with NMM (0.6 g, 6 mM) and the reaction mixture stirred overnight at room temperature. The resulting urea was filtered and the solvent evaporated to give a residue which was chromatographed on Sephadex LH20 eluting with DMF. The major peak eluted at (Ve/Vt – 0.48) and evaporation of the appropriate fractions gave the product **17** after trituration with ether, yielding (2.8 g, 44%), m.p. 207–212°, $\{\alpha\}_D^{23}$ – 15.3 (c = 1, DMF), $R_f(5)$ – 0.3, $R_f(9)$ – 0.7, $R_f(11)$ – 0.8, $Lys_{1.99}Glu_{2.02}$ (Found: C, 52.82; H, 6.00; N, 10.28; Cl, 9.67. $C_{47}H_{63}N_8O_{14}Cl_3$ requires: C, 52.74; H, 5.93; N, 10.47; Cl, 9.94%).

Z-Lys (trans-CO.CH=CH-CO_2H)-Lys (Troc)-Glu (OBu^t)-Gln-NH_2 **18**. The foregoing tetrapeptide derivative **17** (1.4 g, 1.34 mM) was dissolved in DMF (20 ml) and water (4 ml). The pH was adjusted to 10.5 with 1 M NaOH and after the addition of 100 vol H_2O_2 (0.13 ml, 1.34 mM) the pH was maintained at this value by the addition of 1 M NaOH. After 20 min base uptake ceased and the solution was brought to pH7 with 10% citric acid. Evaporation yielded a residue which was triturated with 10% citric acid, the resulting solid being filtered and dried over P_2O_5 giving **18** (1.2 g, 84%), m.p. 206–210°(dec), $\{\alpha\}_D^{23}$ – 13.7° (c = 1, DMF), $R_f(8)$ – 0.1, $R_f(11)$ – 0.6, $Lys_{1.99}Glu_{2.01}$ (Found: C, 49.50; H, 6.10; N, 11.38. $C_{41}H_{59}N_8O_{14}Cl_3$ requires: C, 49.53; H, 5.98: N, 11.27%).

Z-Tyr(Bu^t)-OPh **19**. Z-Tyr(Bu^t)-OH[20] (4.3 g, 11.6 mM) and Ph.OH (1.1 g, 11.6 mM) were dissolved in CH_2Cl_2 (100 ml) and cooled to 0°. Pyridine (0.9 g, 11.6mM) and DCCI (2.4 g, 11.6 mM) were added and the reaction stirred overnight at room temperature. The resulting urea was filtered and the filtrate washed in the usual way. Evaporation of the solvent gave a brownish oil which solidified on trituration with petroleum ether. This material was recrystallised from Et_2O/petroleum ether 60–80° giving **19** (3.2 g, 60%), m.p. 92–95°, $\{\alpha\}_D^{23}$ – 13.1 (c = 1, DMF), $R_f(4)$ – 0.9, $R_f(7)$ – 0.9 (Found;: C, 72.23 H, 6.45; N, 3.36. $C_{27}H_{29}NO_5$ requires: C, 72.46; H, 6.53; N, 3.13%).

Tos O H_2-Tyr(Bu^t)-OPh **20**. The phenyl ester **19** (2.3 g, 5 mM) and Tos.OH.H_2O (0.95 g, 5 mM) were dissolved in DMF (75 ml) and hydrogenolysed for 18 h in the presence of 10% Pd/C (0.23 g). Filtration and evaporation gave the crude salt which was crystallised by the addition of Et_2O giving **20** (2.0 g, 80%), m.p. 216–218° (dec), $\{\alpha\}_D^{23}$ + 27.1° (c = 1, DMF), $R_f(7)$ – 0.4, $R_f(10)$ – 0.8 (Found: C, 63.98; H, 6.37; N, 2.65. $C_{26}H_{31}NO_6S$ requires: C, 64.30; H, 6.43; N, 2.88%).

Z-Lys {trans-CO.CH=CH.CO.Tyr (Bu^t).OPh}-Lys (Troc)-Glu(OBu^t)-Gln-NH_2 **21**. The compound **18** (746 mg, 0.75 mM and HOBt (203 mg, 1.5 mM) were dissolved in DMF (10 ml) and cooled to 0°. DCCI (170 mg, 0.83 mM) was added and the reaction mixtured stirred for 30 min at 0° prior to the addition of the salt **20** (414 mg, 0.83 mM) and NMM (0.091 ml, 0.83 mM). After overnight reaction at room temperature the resulting urea was filtered and the solvent evaporated to yield a gum which crystallised on trituration with 10% citric acid. Reprecipitation from DMF/H_2O gave the required product **21** (800 mg, 82%), m.p. 228–232° (dec), $\{\alpha\}_D^{23}$ – 18.5° (c = 1, DMF), $R_f(6)$ – 0.7, $R_f(8)$ – 0.7, $Lys_{2.02}Glu_{2.05}Tyr_{0.93}$ (Found: C, 55.51; H, 6.13; N, 9.95. $C_{60}H_{80}N_9O_{16}Cl_3$. 0.5 H_2O requires: C, 55.49; H, 6.29; N, 9.71%).

Boc-Gly-Lys {$CO.CH_2.CH_2.CO$.Tyr (Bu^t).OPh}-Lys (Troc)-Glu(OBu^t)-Gln-NH_2 **22**. The fully protected peptide **21** (1.9 g, 1.5 mM) and Tos.OH.H_2O (0.29 g, 1.5 mM) were dissolved in DMF (50 ml) and hydrogenolysed for 8 h in the presence of 5% Pd/C (0.2 g). Work up in the usual way gave the salt as an oil (1.3 g, 64%). This oil, Boc.Gly.OCp[21] (0.39 g, 1.1 mM) and HOBt (0.27 g, 2 mM) were dissolved in DMF (15 ml) at 0° and NMM (0.11 ml, 1 mM) added. After warming to room temperature overnight the solvent was evaporated and the residue triturated with 10% citric acid to give a white solid (1.25 g, 95%). This material was purified in several batches (0.3 g approximately) by dissolving in the minimum of glacial acetic and applying to a column of silica gel (70 g). Elution was initially carried out with a mixture of EtOAc (240 ml), IPA (20 ml), HOAc (6 ml) and H_2O (11 ml) and then with EtOAc (120 ml), IPA (20 ml), HOAc (6 ml) and H_2O (11 ml). Combination of the appropriate fractions and evaporation gave the required product **22** (425 mg, 33%), m.p. 196–200°, $\{\alpha\}_D^{23}$ – 14.5° (c = 1, DMF), $R_f(6)$ – 0.4, $R_f(12)$ – 0.5, $R_f(13)$ – 0.6, $Lys_{1.98}Glu_{2.01}Gly_{1.00}Tyr_{1.00}$ (Found: C, 53.92; H, 6.84; N, 10.69. $C_{59}H_{87}N_{10}O_{17}Cl_3$ requires: C, 53.90; H, 6.67; N, 10.65%).

Boc-Gly-Lys {$CO.CH_2.CH_2.CO$.Tyr (Bu^t).OH}-Lys (Troc)-Glu(OBu^t)-Gln-NH_2 **23**. The preceding peptide 22 (328 mg, 0.25 mM) was dissolved in a mixture of DMF (5 ml) and water (1 ml). DMS (0.73 ml, 10 mM) was added and the pH adjusted to 10.5 with 0.5 M NaOH solution, H_2O_2 100 vol. (0.04 ml) was then added and the pH maintained at 10.5 by the addition of 0.5 M NaOH solution. After 2 h base uptake ceased and the pH was brought to 7 with 10% citric acid prior to evaporation. Trituration with 10% citric acid yielded the required product **23** which was washed with water and ether, then dried yielding (253 mg, 81%), m.p. 198–202° (dec), $\{\alpha\}_D^{23}$ – 9.2° (c = 0.5, DMF), $R_f(6)$ – 0.2, $R_f(13)$ – 0.4, $Lys_{2.07}Glu_{1.93}Gly_{0.98}Tyr_{1.01}$ (Found: C, 51.08; H, 6.93; N, 11.43. $C_{53}H_{83}N_{10}O_{17}Cl_3$.0.5 H_2O requires: C, 51.02; H, 6.70; N, 11.23%).

Scheme 5a

Boc-Gly-Lys {$CO.CH_2.CO$.Tyr (Bu^t).Cys (Acm).Asn.OBu^t}-Lys(Troc)-Glu(OBu^t)-Gln-NH_2 **24**. The peptide acid **23** (248 mg, 0.2 mM) and HOBt (54 mg, 0.4 mM) were dissolved in DMF (5 ml) and cooled to 0°. DCCI (46 mg, 0.22 mM) was added and the reaction mixture stirred at that temperature for 30 min. prior to the addition of the hydrochloride 11 (120 mg, 0.3 mM) and NMM (0.033 ml, 0.3 mM). After overnight reaction at room temperature the resulting urea was removed and the solution evaporated to yield a gum which solidified on trituration with 10% citric acid. This material was purified by chromatography on silica gel (70 g) initially eluting with a mixture of EtOAc (240 ml), IPA (20 ml), HOAc (6 ml) and water (11 ml) and subsequently with EtOAc (120 ml), IPA (20 ml), HOAc (6 ml) and water (11 ml). Pooling of the appropriate fractions as indicated by tlc and evaporation of the solvent gave an oil which crystallised on the addition of Et_2O yielding **24** (134 mg, 42%), m.p. 206–209° (dec), $\{\alpha\}_D^{23}$ – 20.2° (c = 0.5, DMF), $R_f(6)$ – 0.2, $R_f(10)$ – 0.5, $R_f(14)$ – 0.6, $Lys_{1.99}Asp_{0.95}Glu_{2.01}Gly_{1.01}Tyr_{1.04}$ (Found: C, 50.81; H, 7.01; N, 12.58. $C_{67}H_{107}N_{14}Cl_3SO_{21}$ requires: C, 50.83: H, 6.81; N, 12.39). NMR (220 MHz, d_6–DMSO) δ (ppm): 1.25 (s, 9 H; Tyr-C_4H_9 ether); 1.38 {d, 27 H; C_4H_9OCO.Gly,Glu(OC_4H_9), Asn Tyr(Bu^t)-Ala-OPh **25**. The protected fragment 4 (0.87 g, 1 mM) Acm-CH_2): 4.77 (s, 2H; Troc-CH_2).

Scheme 5b

Acm-S.$CH_2.CH_2$.CO-Gly-Glu(OBu^t)-Arg(Adoc)$_2$-Gly-Phe-Phe-Tyr(Bu^t)-Ala-OPh **25**. The protected fragment 4 (0.87 g, 1 mM) and Tos.OH.H_2O (191 mg, 1 mM) were dissolved in DMF (25 ml) and hydrogenolysed for 18 h. in the presence of 10% Pd/C (100 mg). The catalyst was removed and the solvent evaporated giving the salt after trituration with Et_2O (0.88 g, 97%).

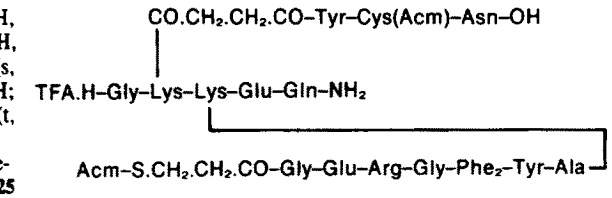
The peptide acid **8** (576 mg, 0.6 mM) and HOBt (162 mg, 1.2 mM) were dissolved in DMF (10 ml); after cooling to 0° DCCI (149 mg, 0.72 mM) was added and the reaction mixture stirred for 30 min. The toluene p-sulphonate obtained above (545 mg, 0.6 mM) and NMM (61 mg, 0.6 mM) were then added and the reaction mixture allowed to warm to room temperature overnight. The resulting urea was removed and the solvent evaporated to yield a pale yellow oil. This oil crystallised on trituration with 1% citric acid and the resulting solid was recrystallised from DMF/Et_2O giving **25** (0.6 g, 59%), m.p. 226–228° (dec), $\{\alpha\}_D^{22}$ – 13.4° (c = 0.5, DMF), $R_f(7)$ – 0.5, $R_f(8)$ – 0.8,

$Arg_{1.01}Glu_{0.96}Gly_{1.98}Ala_{1.04}Tyr_{0.92}Phe_{2.09}$ (Found: C, 62.44; H, 6.88; N, 9.79. $C_{87}H_{118}N_{12}O_{19}S.0.5H_2O$ requires: C, 62.31; H, 7.15; N, 10.02%), NMR (220 MHz, d_6-DMSO) δ (ppm): 1.25 (s, 9 H; Tyr-C_4H_9 ether); 1.35 {s, 9 H; Glu(OC_4H_9)}; 1.42 (d, 3 H; Ala-CH_3); 1.82 (s, 3 H; Acm-CH_3); 4.21 (d, 2 H; Acm-CH_2); 7.4 (t, 2 H; meta protons of phenyl ester).

Acm-S.CH_2.CH_2.CO-Gly-Glu(OBu^t)-Arg(Adoc)$_2$-Gly-Phe-Phe-Tyr(Bu^t)-Ala-OH **26**. The preceding fully protected peptide **25** (340 mg, 0.2 mM) was dissolved in a mixture of DMF (4 ml) and water (1 ml) and DMS (0.58 ml, 8 mM) added. The pH was brought to 10.5 with 0.5 M NaOH solution and maintained at that figure for 2 h after the addition of 100 vol. H_2O_2 (0.03 ml, 0.3 mM). The pH was brought to 7 with 10% citric acid and the solvent evaporated. Trituration with 10% citric acid gave a white solid which was washed with water and ether. The resulting material was purified by gel filtration on Sephadex LH20 eluting with DMF. The fractions corresponding to the major peak (Ve/Vt − 0.45) were pooled and evaporated yielding the required peptide acid **26** (0.25 g, 78%), m.p. 214–218° (dec), $\{\alpha\}_D^{22}$ − 13.4° (c = 0.5, DMF), $R_f(6)$ − 0.2, $R_f(9)$ − 0.7, $Arg_{0.96}Glu_{1.02}Gly_{2.05}Ala_{0.99}Tyr_{0.97}Phe_{2.01}$ (Found: C, 61.40; H, 7.12; N, 10.31. $C_{81}H_{114}N_{12}O_{19}S$ requires: C, 61.11; H, 7.22; N, 10.56%) Nmr (220 MHz, d_6-DMSO) δ (ppm): as compound 25 but with signals due to phenyl ester absent.

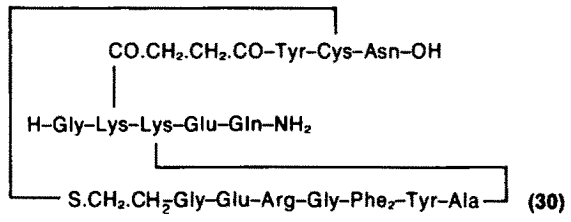
Scheme 6

Z-Gly-Lys {CO.CH_2.CH_2.CO-Tyr(Bu^t)-Cys(Acm)-Asn-OBu^t}-Lys(H_2^+Cl)-Glu(OBu^t)-Gln-NH_2 **27**. The protected peptide **24** (20 mg, 14 μm) was dissolved in a 1/1 mixture of DMF and HOAc (2 ml) and cadmium powder (80 mg) added. After shaking overnight the solution was filtered and the residue washed with DMF, HOAc and water. The filtrate and washings were combined and evaporated to yield a residue which was subjected to gel filtration on Sephadex LH20 eluting with DMF. Fractions corresponding to the major peak (Ve/Vt − 0.50) were pooled and evaporated and the residue dissolved in TFE (4.5 ml) and water (0.5 ml). The pH was adjusted to 1 with 0.1 M HCl in 90% TFE/water and after 5 min the solution evaporated. The resulting residue was solidified by the addition of ether yielding the hydrochloride **27** (17 mg, 85%), m.p. 158–162° (dec), $\{\alpha\}_D^{23}$ − 29.5° (c = 1, DMF), $R_f(10)$ − 0.2, $R_f(15)$ − 0.1, $R_f(16)$ − 0.6, $Lys_{1.94}Asp_{0.96}Glu_{2.02}Gly_{1.06}Tyr_{1.02}$, Nmr (220 MHz, d_6-DMSO) δ (ppm): 1.25 (s, 9 H; (Tyr-C_4H_9); 1.38 (d, 27 H; C_4H_9.O.CO.Gly + Glu-OC_4H_9 + Asn-OC_4H_9); 1.87 (s, 3 H; Acm-CH_3); 4.05–4.60 (m, 9 H; 7 α-CH + Acm-CH_2).

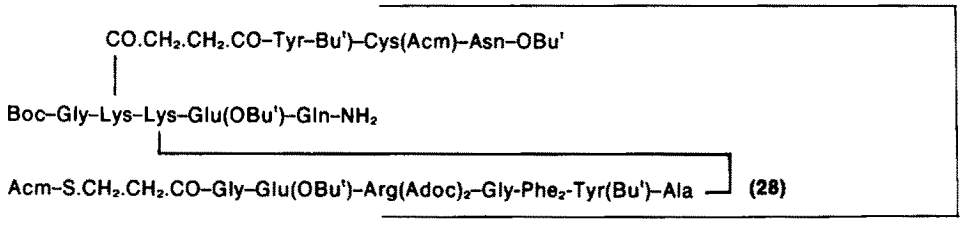


(29)

The fully protected peptide **28** (10 mg, 3.3 μM) was dissolved in a mixture of 90% trifluoroacetic acid (2.6 ml) and ethane-1, 2-dithiol (16 μl, 165 mM) under nitrogen. After 4h storage in the dark, ether was added and a white precipitate collected by centrifugation. This material was subjected to ion exchange chromatography on carboxymethyl cellulose (CM32) equilibrated with sodium acetate (0.024 M) at pH 3.3 at 4°. A linear gradient to 0.1M NaCl was run and the major fractions collected and combined. The combined fractions were then applied to Sephadex G15 eluting with 50% acetic acid at 4°. The major peak (Ve/Vt − 0.51) was isolated by pooling of the appropriate fractions and lyophilisation giving **29** (3.2 mg, 48%), $\{\alpha\}_D^{22}$ − 61° (c = 0.01, 50% CH_3CO_2H), $R_f(17)$ − 0.4, $R_f(18)$ − 0.1, $E^{1\%} = 0.09$, $Lys_{2.14}Arg_{0.87}Asp_{0.98}Glu_{3.12}Gly_{2.90}Ala_{1.06}Tyr_{1.98}Phe_{1.95}$, UV λ_{max} (0.2 M HOAc) 275 nm, NMR (360 MHz, d_4-HOAc) δ (ppm): 1.44 (d, 3 H; Ala-CH_3), 1.20–1.75 (m, 22H; 2 × Glu β CH_2's + Gln β CH_2's + 2 × Lys, β, γ and δ CH_2's + Arg β and γ CH_2's); 2.16–2.53 (m, 10 H; 2 × Glu γ CH_2's + Gln γ CH_2 + succinyl CH_2-CH_2), 2.50 (t, 2 H) and 2.70 (t, 2 H) (Acm-SCH_2CH_2CO); 2.82–3.34 (m, 14 H; 2 × Lys ϵ-CH_2 + 2 × Tyr CH_2 + 2 × Phe CH_2 + Arg δ-CH_2), 3.90–4.94 (m, 11 H; 2 × Lys α-H's + Ala α-H + 2 × Glu α-H's + Ala α-H + 2 × Glu α-H's + Arg α-H + Gln α-H + 2 × Tyr α-H's + 2 × Phe α-H's) 4.07 (m, 6 H; 3 × Gly CH_2's), 4.39 (s, 4 H; 2 × Acm-CH_2's); 6.76 (m, 4H) 7.05 (m, 4H)(2 × Tyr C_6H_4); 7.08–7.25 (m, 10 H; 2 × Phe C_6H_5).



(30)



(28)

The protected peptide acid **26** (20 mg, 12.6 μM) and HOBt (4 mg, 25.3 μM) were dissolved in DMF (2 ml) and cooled to 0° prior to the addition of DCCI (3 mg, 15.7 μM). After stirring for 30 min at 0° the salt **27** (30 mg, 12.6 μM) was added followed by NMM (1.26 μl, 12.6 μM) in the minimum volume of DMF. The reaction mixture was stirred for 48 h at room temperature and then subjected to gel filtration on Sephadex LH60 eluting with DMF. The fractions corresponding to the major peak at (Ve/Vt − 0.61) were pooled, and evaporated to give a residue which solidified on trituration with ether and water yielding **28** (12 mg, 30%), m.p. 221–225° (dec), $\{\alpha\}_D^{23}$ − 15.4° (c = 0.8, DMF), $R_f(10)$ − 0.5, $R_f(15)$ − 0.6, $Lys_{2.13}Arg_{0.94}Asp_{0.94}Glu_{3.02}Gly_{2.91}Ala_{1.13}Tyr_{1.89}Phe_{2.04}$ (Found: C, 55.42; H, 7.28; N, 11.86. $C_{145}H_{218}N_{26}O_{37}S_2.8\ H_2O$ requires: C, 55.71; H, 7.03; N, 11.65%). Nmr (360 MHz, d_6-DMSO) δ (ppm): 1.21 (s, 9 H; B chain Tyr-C_4H_9); 1.25 (s, 9 H: A chain Tyr-C_4H_9), 1.36 (broad s, 36 H; C_4H_9.O.CO.Gly + 2 × Glu-OC_4H_9 + Asn-OC_4H_9): 1.82 (s, 3 H; B chain Acm; CH_3); 1.86 (s, 3 H; A chain Acm-CH_3).

The preceding peptide (2 mg, 1.07 μM) was dissolved in 50% HOAc (0.6 ml) and added over 9 min to a solution containing iodine (9 mg, 36.2 μM) in HOAc (2.13 ml), H_2O (0.72 ml) and 0.1 M HCl (6.4 μl). The reaction mixture was stirred for a further 8 min and then decolourised by the addition of 1M $Na_2S_2O_3$ solution (74 μl) and 0.5 M NaOAc solution (160 μl). The solvent was evaporated and the residue subjected to gel filtration on Sephadex G15 eluting with 50% HOAc at 4°. The fractions corresponding to the major peak (Ve/Vt − 0.50) were pooled and evaporated. The resulting product was further purified by ion exchange chromatography at 4° on carboxymethyl cellulose (CM32) initially equilibrated with NaOAc (0.024 M) at pH 3.3. A linear gradient of NaCl was run from 0 to 0.1 M and again the fractions corresponding to the major peak pooled. The combined fractions were then desalted by running on Sephadex G15 eluting with 50% HOAc and the resulting fractions of the major peak (Ve/Vt − 0.51) were pooled and lyophilised giving **30** (0.8 mg, 40%), $\{\alpha\}_D^{22}$ − 77° (c = 0.027, 50% HoAc), $R_f(17)$ − 0.4 $R_f(20)$ − 0.1, $E^{1\%}$ =

0.11, $Lys_{2.16}Arg_{0.93}Asp_{0.99}Glu_{3.06}Gly_{2.86}Ala_{1.16}Tyr_{1.93}Phe_{1.93}$ UV λ_{max} (0.2M, HOAc) 275, Nmr (360 MHz d_4-HOAc) δ (ppm): 1.47 (d, 3H; Ala-CH_3); 1.16–1.90 (m, 22H; 2 × Glu β CH_2's + Gln β CH_2 + 2 × Lys, β, λ and β CH_2's + Arg β and λ CH_2's); 2.18–2.56 (m, 10H; 2 × Glu γ CH_2's + succinyl CH_2-CH_2); 2.58 (t, 2H) and 2.76 (t, 2H) (-S-S-CH_2CO); 2.82–3.36 (m, 14H; 2 × Lys ϵ-CH_2's + 2 × Tyr CH_2 + 2 × Phe CH_2 + Arg δ-CH_2); 3.97–4.97 (m, 17H; 11 α-H's + 3 × Gly CH_2's); 6.79 (m, 4H) and 7.04 (m, 4H) (2 × Tyr C_6H_4); 7.14–7.32 (m, 10H; 2 × Phe C_6H_5).

Acknowledgements—We thank Prof. J. Gliemann and Dr. O. Sonne of the Institute of Medical Physiology, University of Copenhagen for the determination of the biological potency and binding activity of our analogue. Also we thank the Science Research Council for a CASE studentship to one of us (G. H.) which was supported by Reckitt and Colman Ltd.

REFERENCES

[1]J. Meienhofer, E. Schnabel, H. Bremer, O. Brinkoff, R. Zabel, W. Stroka, H. Klostermeyer, D. Brandenburg, T. Okuda and H. Zahn, *Z. Naturforsch.* **186**, 1120 (1963).
[2]P. G. Katsoyannis, A. Tometsko, K. Fukuda, K. Suzuki and M. Tilak, *J. Am. Chem. Soc.* **86**, 930 (1964).
[3]K.-T. Kung, Y.-C. Du, W.-T. Huang, C.-C. Chen, L.-T. Ke, S.-C. Hu, R.-Q. Jiang, S.-Q. Chu, C.-I. Niu, J.-Z. Hsu, W.-C. Chang, L.-L. Cheng, H.-S. Li, Y. Wang, T.-P. Loh, A.-H. Chi, C.-H. Li, P.-T. Shi, Y.-H. Yieh, K.-L. Tang, and C.-Y. Hsing, *Sci. Sinca* **14**, 1710 (1965).
[4]P. Sieber, B. Kamber, A. Hartmann, A. Johl, B. Riniker and W. Rittel, *Helv. Chim. Acta* **57**, 2617 (1974).
[5]T. L. Blundell, G. G. Dodson, D. C. Hodgkin and D. A. Mercola, *Adv. in Protein Chemistry* **26**, 279 (1972).
[6]*Methoden der Organischen Chemie, Synthese von Peptiden.* Houben-Weyl (Edited by E. Wünsch), Georg Thieme Verlag, Stuttgart.
[7]I. J. Galpin, P. M. Hardy, G. W. Kenner, J. R. McDermott, R. Ramage, J. Seely and R. G. Tyson, *Tetrahedron* **35**, 2577 (1979).
[8]G. Jager and R. Geiger, *Chem. Ber.* **103**, 1727 (1970).
[9]R. B. Woodward, K. Hausler, J. Gosteli, P. Naegeli, W. Oppolzer, R. Ramage, S. Ranganathen and H. Vorbrüggen, *J. Am. Chem. Soc.* **88**, 852 (1966).
[10]T.B. Windolz and D. B. R. Johnston, *Tetrahedron Letters* 2555 (1967).
[11]I. J. Galpin, G. W. Kenner, B. A. Morgan, P. Noble and R. Ramage, *Tetrahedron* **37**, 3031 (1981).
[12]J. Beecham, G. Dupuis, F. M. Finn, H. T. Storey, C. Yanaihara, N. Yanaihara and K. Hofmann, *J. Am. Chem. Soc.* **93**, 5526 (1971).
[13]P. Marbach and J. Rudinger, *Helv. Chim. Acta* **57**, 403 (1974).
[14]R. Roeske, *J. Org. Chem.* **28**, 1251 (1963).
[15]I. J. Galpin, G. W. Kenner, S. R. Ohlsen, R. Ramage, R. C. Sheppard and R. G. Tyson, *Tetrahedron* **35**, 2785 (1979).
[16]B. Riniker, B. Kamber and P. Sieber, *Helv. Chim. Acta* **58**, 1086 (1975).
[17]B. Bezas and L. Zervas, *J. Am. Chem. Soc.* **83**, 719 (1961).
[18]H. M. Walton and C. S. Nevin, *U.S. Patent* 3,238,162.
[19]G. Hancock, I. J. Galpin and B. A. Morgan, *Tetrahedron Letters* 000 (1982).
[20]E. Wünsch and A. Zwick, *Chem. Ber.* **99**, 105 (1966).
[21]M. Itoh, *Chem. Pharm. Bull. Japan* **18**, 784 (1970).
[22]R. Geiger, K. Geisen, G. Regitz, W. Pfaff and H. D. Summ, *Mol. Endrocrinol. Proc.* **27** (1977).
[23]K. B. Mathur, H. Klostermeyer and H. Zahn, *Hoppe-Seyler's Z. Physiol. Chem.* **346**, 60 (1966).
[24]G. W. Anderson, J. E. Zimmerman, F. M. J. Callaghan, *J. Am. Chem. Soc.* **86**, 1839 (1964).
[25]E. Schnabel, *Annalen* **702**, 188 (1967).
[26]J.S. Morley, *J. Chem. Soc.* (*C*) 2410 (1967).
[27]H. Yajima, H. Watanabe and M. Okamoto, *Chem. Pharm. Bull. Japan* **19**, 2185 (1971).