The Use of Crown Ethers in Peptide Chemistry. Part 1. Syntheses of Amino Acid Complexes with the Cyclic Polyether 18-Crown-6 and their Oligomerisation in Dicyclohexylcarbodi-imide-containing Solutions

Paolo Mascagni,* Carolyn B. Hyde, Mario A. Charalambous, and Kevin J. Welham
Department of Pharmaceutical Chemistry, School of Pharmacy, University of London, London WC1N 1AX

The synthesis of amino acid complexes with the cyclic polyether 18-crown-6 and their solubility properties in organic solvents are described. Oligo homo-amino acid peptides have been prepared using the crown ether complexes and dicyclohexylcarbodi-imide as coupling agent. The mechanism leading to the formation of the oligopeptides has been discussed and proved to involve the transferring of one N-H proton from the crown ether complex to the carbodi-imide nitrogen.

Since their discovery by Pedersen,[1] crown ethers have found increasing application in the fields of organic, inorganic, and analytical chemistry.[2] Their most striking characteristic is the capacity to form stable complexes with inorganic salts, which then become soluble in various organic solvents including nonpolar ones.[1,2] This ability is not restricted to inorganic species only; charged amines have also been shown to enter the cavity formed by the donor atoms (N or O) of crown-type ligands.[1,2] In the latter case H-bonds as well as ion–dipole interactions have been considered responsible for the stability of such complexes.[3] These observations led some authors[4] to exploit the complexation ability of crown ethers for selective acylation of secondary amines in the presence of primary ones.

The use of crown ethers as protecting groups suggested by this experiment is, in our view, extendable to other classes of compounds such as amino acids; thus peptide synthesis, both in solution and solid phase, implies selective masking of the latter at the amino group. Should this approach be feasible, *in situ* protection could be performed which would result in a substantial shortening of the overall synthetic process.

Recent results seem to substantiate this approach.[5] Thus, following the original idea of Cram and his co-workers,[6] crown ethers bearing thiofunctions were prepared and shown to function as enzyme model in the synthesis of peptides.[5]

We report here the synthesis of amino acid oligomers as part of a wider study on the applications of crown-type ligands to the field of peptide and macromolecular synthesis.

The mechanism of oligomerisation is proposed by analysing the behaviour towards dicyclohexylcarbodi-imide (DCC) of several amine and amino acid complexes with crown ether. DCC is commonly used as coupling agent in peptide synthesis and its mode of action is shown in Scheme 1.[7]

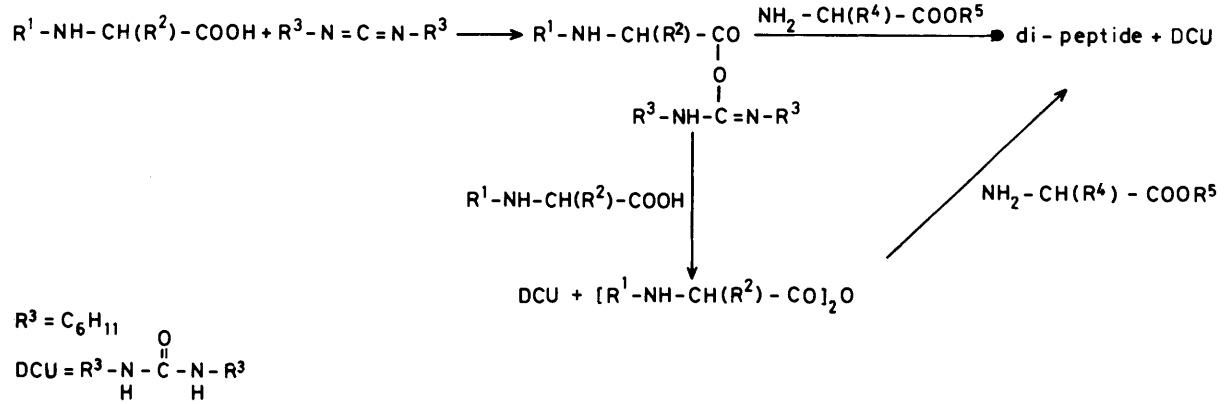
Experimental

18-Crown-6 was purchased from Aldrich Chemical and used without further purification. M.p.s are uncorrected. One- and two-dimensional n.m.r. spectra were taken on a Varian XL-300 instrument; chemical shifts refer to internal tetramethylsilane as standard.

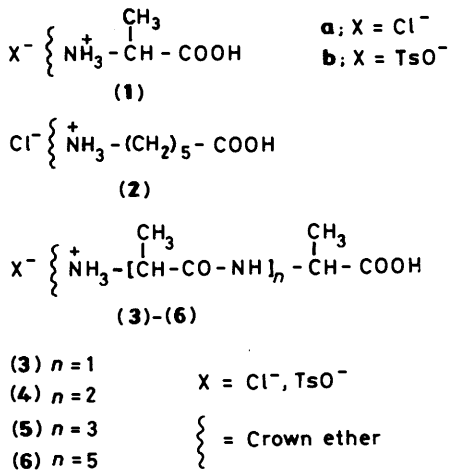
Fast-atom bombardment mass spectrometry was performed on a VG Analytical ZAB-IF double-focusing mass spectrometer. The samples were applied to the probe tip in a thioglycerol matrix and bombarded by 8 keV Xenon atoms. Full scan spectra were recorded in *ca.* 7 s.

Alanine Hydrochloride Crown Ether Complex (**1a**).—Alanine (1 equiv.; 1 g, 1.12 × 10^{-2} mol) was dissolved in water (5 ml) and HCl (1.1 equiv.; 0.4502 g, 1.06 ml) was added. The solution was stirred for 0.5 h and then lyophilised to give alanine hydrochloride in 100% yield. The hydrochloride (1 equiv.; 1 g, 7.96 mmol) was suspended in chloroform (5—10 ml) and 18-crown-6 (1 equiv.) added. The solution was stirred until it became clear, then the solvent was evaporated off to give a powder in 97% yield, m.p. 149—150 °C (Found: C, 46.3; H, 8.3; N, 3.5. $C_{15}H_{32}ClNO_8$ requires C, 46.2; H, 8.27; N, 3.6%); M^+ (less Cl^-) 354; δ($CDCl_3$) 11 (1 H, br), 7.11 (3 H, br), 4.42 (1 H, m), 3.59 (30 H, s), and 1.5 (3 H, *J* 8.8 Hz).

Alanine Tosylate 18-Crown-6 Complex (**1b**).—The tosylate complex was made in a similar way, but toluene-*p*-sulphonic



Scheme 1.



acid was used. The complex was recrystallised from ethanol–ethyl acetate mixtures in 77% yield, m.p. 123—125 °C (Found: C, 50.0; H, 7.5; N, 2.8. $C_{22}H_{39}NO_{11}S$ requires C, 50.3; H, 7.5; N, 2.7%); δ($CDCl_3$) 7.8 (2 H, d, *J* 8.3 Hz), 7.27 (3 H, br), 7.12 (2 H, d, *J* 8.3 Hz), 4.25 (1 H, br), 3.65 (34 H, br), 2.31 (3 H, s), and 1.6 (3 H, d, *J* 6.9 Hz).

6-Aminohexanoic Acid Hydrochloride Complex (**2**).—The synthesis was identical to that of (**1a** and **b**) and gave 88% of the complex, m.p. 105—107 °C [Found: C, 50.0; H, 8.8; N, 3.3. $C_{18}H_{38}ClNO_8$ requires C, 50.0; H, 8.9; N, 3.2%); δ($CDCl_3$) 9.76 (1 H, br), 6.86 (3 H, br), 3.40 (29 H, br), 2.73 (2 H, m), 2.53 (2 H, t, *J* 8 Hz), 1.67 (2 H, m), 1.55 (2 H, m), and 1.43 (2 H, m).

Alanine Dipeptide Complex (**3**).—This had m.p. 192—194 °C; M^+ (less Cl^-) 425; δ([$^2H_6$]DMSO) 8.71 (1 H, d, *J* 8.5 Hz), 8.1 (3 H, br), 4.26 (1 H, m), 3.83 (1 H, m), 3.52 (25 H, br), 1.38 (3 H, d, *J* 7.1 Hz), and 1.33 (3 H, d, *J* 7.0 Hz).

Alanine Tripeptide Complex (**4**).—This has M^+ (less Cl^-) 496; δ($CDCl_3$) 10.37 (1 H, d, *J* 9.4 Hz), 10.04 (1 H, d, *J* 7.5 Hz), 7.15 (3 H, br), 4.51 (1 H, m), 4.46 (1 H, m), 3.7 (26 H, br), 3.35 (1 H, m), and 1.57 (9 H, three partially overlapping d).

Alanine Tetrapeptide Complex (**5**).—This has M^+ (less Cl^-) 567; δ([$^2H_6$]DMSO) 8.72 (1 H, d, *J* 7.7 Hz), 8.51 (1 H, d, *J* 8.7 Hz), 8.27 (1 H, d, *J* 9.6 Hz), 4.37 (1 H, m), 4.27 (1 H, m), 4.19 (1 H, m), 3.85 (1 H, br), 3.55 (27 H, br), and 1.45—1.22 (12 H, partially overlapping d).

Reaction between Vilsmeier Reagent[8] *and Alanine Hydrochloride Complex* (**1a**).—The Vilsmeier reagent was prepared by adding PCl_5 (3 g) in small portions to an excess of DMF at 0 °C with stirring.[9] A precipitate formed which was the Vilsmeier reagent. The complex (**1a**) (100 mg) was dissolved in $CDCl_3$ (3 ml) and the solution cooled to −40 °C. An aliquot portion of Vilsmeier reagent was filtered, washed with diethyl ether, and immediately transferred, with the aid of a glass spatula, to the $CDCl_3$ solution. It was not possible to transfer known quantities of the Vilsmeier reagent since the latter is extremely hygroscopic and decomposes very rapidly in the presence of moisture. The $CDCl_3$ solution was allowed to equilibrate for 1 h at −40 °C before a sample was taken out for n.m.r. analysis.

Results

(a) *Alanine Hydrochloride Complex with* 18-*Crown*-6.—Suspensions of alanine hydrochloride salt in chloroform readily turned into clear solutions upon addition of one equivalent of the cyclic polyether 18-crown-6. The crystals obtained after solvent removal and recrystallisation from the appropriate solvent were shown by mass spectrometry, ^1H n.m.r., and elemental analysis to be those of the amino acid salt complex with the crown ether, (**1a**).

The reactivity towards DCC of the amino acid thus blocked was tested in acetonitrile and chloroform.

When one equivalent of DCC was added to a solution of (**1a**) (100 mg) in acetonitrile a large precipitate readily formed. This was isolated (33 mg) and shown to be dicyclohexylurea (DCU) by comparison with an authentic sample. From the reaction mixture after an overnight of stirring at room temperature alanine dipeptide complex (**2**) (15 mg) was isolated. Analysis of the mother liquor revealed the following. (i) Based on the lack of i.r. absorption at 2 120 cm^{-1}, it was concluded that virtually all DCC had been consumed. (ii) The pH of the solution was 2.9,* identical to that of the mixture prior to addition of DCC. (iii) Solid (35 mg) was isolated after extraction with water and lyophilisation. FAB mass spectrometry and n.m.r. showed that this solid contained *ca.* 80% by weight of the alanine tetrapeptide (**5**). (iv) Small amounts of peptides of different length were seen in the spectrum of the reaction residue. (v) The dipeptide and the tetrapeptide thus isolated accounted for *ca.* 95% of the initial amino acid.

When the reaction was repeated in chloroform solutions different results were obtained. This is illustrated by the following example. Complex (**1a**) (50 mg) was dissolved in $CDCl_3$ (0.5 ml). The solution was then treated with one equivalent of DCC and the reaction followed by ^1H n.m.r. over a period of three weeks. Representative spectra of this time-dependent experiment are illustrated in Figure 1.

The appearance of doublets in the δ 7—11 range confirmed the formation of peptide bonds. Subsequent to the addition of DCC and vigorous shaking of the n.m.r. sample, the spectrum (Figure 1A) had a signal at δ *ca.* 9.7 which further analysis showed to be the dimeric species of the alanine complex. Three other doublets of lesser intensity were seen between δ 7 and 11. Occasional runs in the following five days indicated that the composition of the mixture was still changing (Figure 1B and C). No further changes were seen in the shape of the spectrum after this time (Figure 1D). The reaction was assumed to have gone to completion. Separation of dicyclohexylurea and treatment of the oily residue with (i) water and (ii) acetonitrile yielded alanine tripeptide complex (10 mg) with crown ether (**4**). The analysis of the remaining peptides was carried out by n.m.r. and mass spectrometry. A two-dimensional correlation spectrum of the reaction mixture revealed a number of couplings between the NH and α-protons, consistent with the presence of several oligomeric species of alanine. FAB mass spectra of the reaction residue were more difficult to interpret. Thus small amounts of by-products, and in particular dicyclohexylurea, appeared to discriminate against the more abundant peptides. However upon repetition of the reaction some peptides were positively identified: they were the tri- [(**4**); M^+ less Cl^-, 496], tetra- [(**5**); M^+ less Cl^-, 567], and, hexa-peptide [(**6**); M^+ less Cl^-, 709] of alanine complex with crown ether.

(b) *Alanine Tosylate Complex with* 18-*Crown*-6.—The complex of alanine tosylate with the crown ether, (**1b**), was prepared in a fashion identical to that described for the corresponding hydrochloride complex. The effects caused by the substitution of the counteranion on the reactivity of the amino acid complexes were investigated in the following manner.

* A portion of the solution was diluted with water (2 vol.) and the pH measured using a pH-meter.

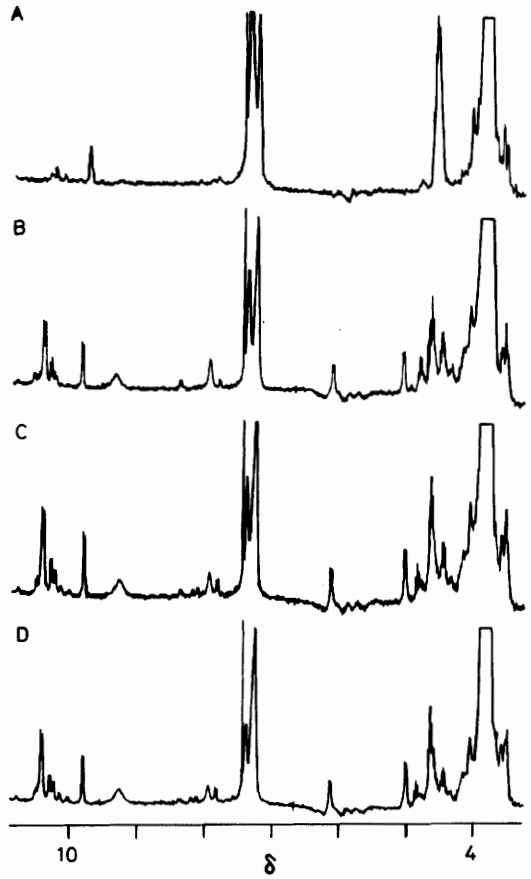


Figure 1. ^{1}H N.m.r. spectra of a reaction of (**1a**) in DCC containing $CDCl_3$ solution: A, after addition of 1 equivalent of DCC; B, after 1 h from addition of DCC; C, after 24 h from addition of DCC; D, after 5 days from beginning of reaction

The complex (**1a** or **b**) (100 mg) was dissolved in acetonitrile (5 ml) containing 1 equivalent of DCC. After 0.5 h dicyclohexylurea was collected by filtration, dried, and weighed. The clear solution thus obtained was stirred for a further 24 h at room temperature, after which time the solvent was evaporated; the oily material thus obtained was taken up in chloroform and extracted with water. The water extracts were then lyophilised and checked by n.m.r for their amino acid content. The chloroform layers were also checked and shown not to contain any unchanged amino acid or derivatives. Following this procedure we were able to conclude that, although the two complexes produced oligomeric species in virtually the same relative ratio, the tosylate was more stable in the presence of DCC. Thus the n.m.r. spectra of the water extracts showed the presence of di- and tri-peptide in 2:1 ratio, with unquantifiable traces of higher peptides, and *ca.* 25% of unchanged (**1b**) as compared to only 5% of the monomeric hydrochloride complex.* Furthermore, *ca.* 35% of the expected urea was obtained from the reaction involving the tosylate compound, whereas 58% urea was recovered from the hydrochloride complex reaction.

(c) N-*Methylamine Hydrochloride Complex with* 18-*Crown*-6.—When inorganic or organic salts are solubilised in organic solvents, the counteranion is present in solution as an active, 'naked' anion which is not solvated. Because of this characteristic the electron-donating ability of the anion is increased, *i.e.* the nucleophilicity and basicity of the anion are enhanced.

Fearing that the complex counteranion in its highly activated state may participate in the reaction mechanism, we investigated the behaviour in chloroform of dicyclohexylcarbodi-imide-containing solutions of (1) *N*-methylamine hydrochloride complex with crown ether, (2) the latter and benzoic acid, and (3) benzoic anhydride plus the methylamine complex. Here is a summary of the results obtained from the three reactions.

When equimolar amounts of the *N*-methylamine hydrochloride complex and DCC were dissolved in chloroform no dicyclohexylurea precipitated from the solution even after 24 h stirring at room temperature, indicating that the two reagents were stable in solution in the presence of one another. That the 'naked' chloro ion was not reacting in this condition was also confirmed by the i.r. spectra taken of the reaction mixture. Thus the intensity of the DCC absorption at 2 120 cm^{-1} did not change upon addition of the methylamine complex, nor during the 24 h period of stirring at room temperature.

Surprisingly the addition of benzoic acid to a chloroform solution of *N*-methylamine hydrochloride complex and DCC did not result in the complete conversion of benzoic acid into the amide derivative. Thus after 48 h of vigorous stirring at room temperature the solvent was removed and the ^{1}H n.m.r. spectrum of the oily product thus obtained showed the presence of *N*-methylbenzamide and benzoic anhydride (formed during the reaction) in 1:14 ratio.

Identical results were obtained upon treatment of chloroform solutions of the complex and DCC with benzoic anhydride. The latter is the condensation product of two molecules of benzoic acid. Such a condensation is often carried out with the aid of DCC and in peptide chemistry the amino acid anhydride is regarded as a possible active intermediate of the coupling step mediated by DCC (Scheme 1).

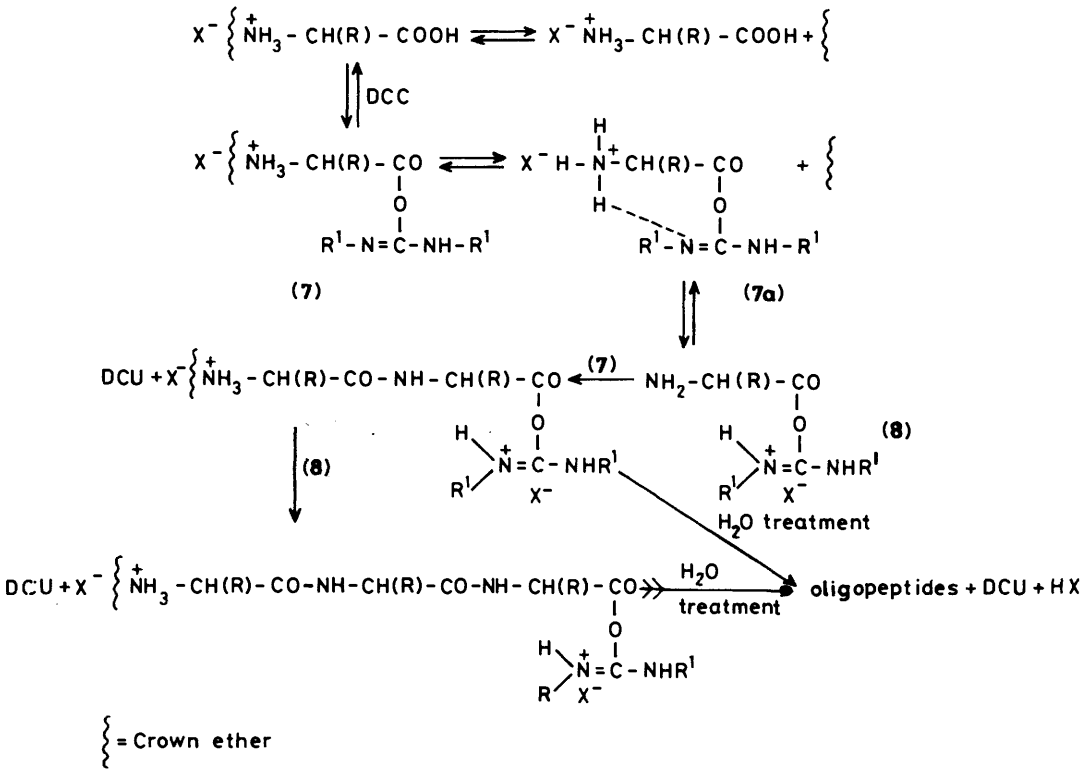
Following the experiments with *N*-methylamine it became apparent that the polymerisation process seen in the case of the alanine complexes takes place *via* an intermediate involving the amino group, the carboxylic group, and DCC and that the former two groups must belong to the same molecule.

(d) 6-*Aminohexanoic Acid Complex with* 18-*Crown*-6.—In order to confirm the latter conclusions and to establish if the number of covalent bonds separating the amino and carboxylic moieties plays any role at all in the reactivity of these amino acid complexes, the behaviour of 6-aminohexanoic acid complex with crown ether was investigated in the reaction conditions which yield oligomerisation. The complex (**2**) was prepared from suspensions of the amino acid salt and crown ether and shown to be homogeneous by elemental analysis and ^{1}H n.m.r. Complex (**2**) and DCC in equimolar amounts were thoroughly mixed in chloroform or acetonitrile and the solutions were stirred for 24 h. Only traces of precipitated dicyclohexylurea were collected at the end. Typical amounts corresponded to 10% of the urea expected from complete conversion of DCC. The amino acid components of the reaction mixture were then extracted in water and after lyophilisation the extracts were checked for 6-aminohexanoic acid oligomers. The n.m.r. spectrum revealed that the water extracts contained the majority of the amino acid molecules still in the intact form: only 5% of the latter had been converted into the dipeptide.

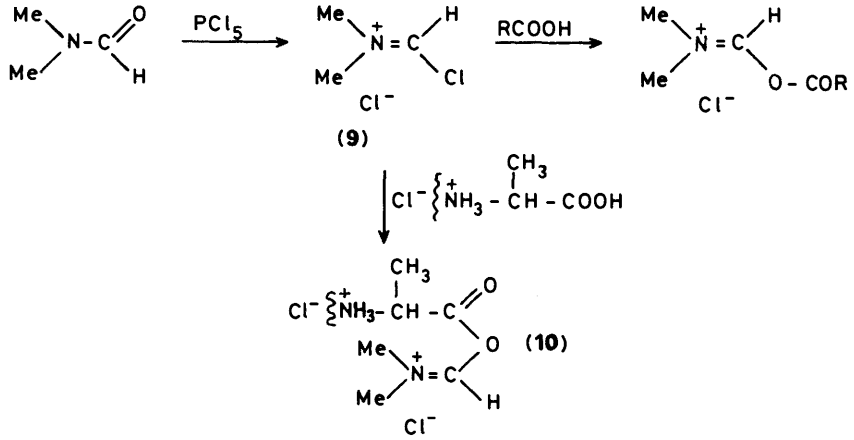
Discussion

The coupling reaction between the amino and carboxylic groups to form the amide bond requires the presence in solution of the amino component as a free base. In order to explain the

* The rate of oligomerisation appeared to be different when the solutions were vigorously stirred. The reaction carried out in $CDCl_3$ was slower when compared with that of identical mixtures stirred in a flask with the aid of magnetic bars.



Scheme 2.



Scheme 3.

mechanism of polymerisation of the amino acid complexes shown here, it is necessary to find out which is the mechanism that produces the free amino end, out of the RNH_3^+ salt. The results seem to indicate that an intramolecular rearrangement is involved in the latter mechanism. Furthermore, the data relative to the methylamine complex rule out the presence in solution of the equilibrium $NH_3^+ \rightleftharpoons NH_2 + HCl$ with DCC and/or traces of water acting as catalyst. Thus, should the contrary apply, a complete conversion of benzoic acid (or benzoic anhydride) into the corresponding amide would take place. A possible reaction mechanism that takes into account the experimental data is shown in Scheme 2 where the amino acid complex and the amino acid complex adduct with DCC are in equilibrium with the corresponding uncomplexed ion pairs. Because of the close proximity of the NH_3^+ to the DCC nitrogen in the adducts (**7**) and (**7a**), a hydrogen-bond bridge can be formed between these two centres. This results in a redistribution of the positive charge, which is no longer concentrated on the amino acid nitrogen, and consequently a weakening of the electrostatic interaction between the counteranion and NH_3^+. The equilibrium between (**7a**) and the new species (**8**), in which the chloro atom forms an electrostatic interaction with the DCC nitrogen, provides the necessary free amino group for the coupling reaction. Supporting evidence for this hypothesis came from the reaction between (**1a**) and the Vilsmeier reagent[8] (**9**). The latter is known to form adducts[10] of the type of those between carboxylic acids and DCC (Scheme 3). Furthermore, the presence of the positive charge on the $C{=}N^+$ moiety would

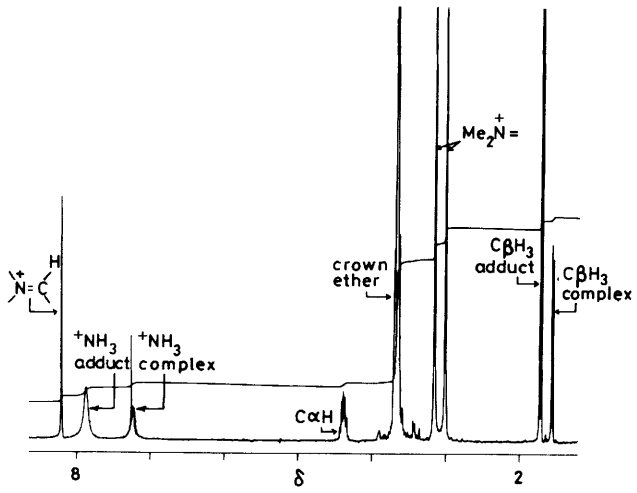


Figure 2. ^{1}H N.m.r. spectrum of a mixture of (**1a**) and Vilsmeier reagent in $CDCl_3$

prevent hydrogen bonding and hence polymerisation, with the assumption that the proposed mechanism holds. And indeed addition of alanine hydrochloride complex to $CDCl_3$ solutions containing freshly prepared[9] Vilsmeier reagent produced the wanted adduct (**10**) but no polymerisation took place as indicated by n.m.r. In Figure 2 the ^{1}H n.m.r. spectrum of the latter solution after stirring overnight at room temperature shows two sets of peaks attributed to the free amino acid complex and to the adduct (**10**). No traces of polymeric form could be detected in the spectrum.

In the light of these conclusions the experimental results presented above are readily explained. Thus the limited reactivity of the 6-aminohexanoic acid complex towards oligomerisation follows the almost total lack of hydrogen bonding between the nitrogen on DCC and the amino group in the complex. The spacer group $(CH_2)_4$ introduced in the amino acid prevents close contact between the acceptor and donor of the hydrogen-bond thus stabilising the adduct (**7c**).

Identical reasons apply to the *N*-methylamine case. Here the lack of covalent bonding between the amino and the carboxylic groups does not allow the DCC to come close to the NH_3^+ and hence deprotonation of the amine cannot take place.

On account of its larger size, 'naked' tosylate ion should display greater stability than that of the smaller chloride ion in solvents of low polarity. This characteristic should make the tosylate ion in (**1b**) less readily available for the formation of the species (**8**) thus explaining the presence of larger amounts of unchanged monomer found in the reaction mixtures of the tosylate complex.

Conclusions

With the objective of establishing the scope and generality of the use of crown ethers as non-covalent blocking groups in peptide synthesis, we have investigated the mode of action of amino acid–crown ether complexes in DCC-containing solutions. The results presented indicate that oligomerisation of α-amino acid complexes takes place in solution and proceeds *via* the cyclic intermediate (**7a**). A hydrogen bond between the amino group nitrogen and the DCC nitrogen ultimately causes the 'deprotection' of the former and its coupling to the carboxylate of a second amino acid unit. Upon repetition of these same steps oligomerisation is achieved.

Because of the nature of their mode of action, the reactivity of these complexes is achieved at the price of selectivity. Thus, using the experimental conditions here employed, it seems difficult to control the reaction and hence produce selective coupling between amino acids of different kinds. On the other hand the elucidation of the reaction mechanism clearly indicates that coupling reagents, having structural characteristics different from those of DCC, can be used, in principle, for the selective formation of peptide bonds.

References

1 C. J. Pedersen, *J. Am. Chem. Soc.*, 1967, **89**, 2495.
2 M. Hiraoka, 'Crown Compounds,' Elsevier, Amsterdam, 1982.
3 C. J. Pedersen, *J. Am. Chem. Soc.*, 1967, **89**, 7017.
4 A. G. H. Barett and J. C. A. Lana, *J. Chem. Soc., Chem. Commun.*, 1978, 471.
5 S. Sasaki, M. Shionoya, and K. Koga, *J. Am. Chem. Soc.*, 1985, **107**, 3371.
6 Y. Chao and D. J. Cram, *J. Am. Chem. Soc.*, 1976, **98**, 1025.
7 M. Bodanszky, Y. S. Klausher, and M. A. Ondetti, 'Peptide Synthesis,' Wiley Interscience, New York, 1976.
8 A. Vilsmeier and A. Haack, *Ber. Dtsch. Chem. Ges.*, 1927, **60**, 119.
9 D. R. Hepburn and H. R. Hudson, *Chem. Ind. (London)*, 1974, 664.
10 T. Fujisawa, T. Mori, S. Tsuge, and T. Sato, *Tetrahedron Lett.*, 1983, **24**, 1543.

Received 22*nd May* 1986; *Paper* 6/993