2-Methyl-8-oxo-8*H*-pyrano[3,2-*g*]benzoxazole-7-carboxylic Acid (IV)——After a mixture of 1 g of II and 10 ml of tetraphosphoric acid had been heated at 100°—110° for 5 hr, the sticky solution was poured into about 100 ml of an ice water. After standing at between 5° and 10° for 24 hr, separating crystals were collected by suction, washed with H_2O, dried and recrystallized from EtOH to give the product (IV) as light yellow needles (0.7 g), mp 248° (decomp.), which was identical with 2-methyl-8-oxo-8*H*-pyrano[3,2-*g*]-benzoxazole-7-carboxylic acid on the admixed melting point test.

N-(2-Pyridyl)-2-methyl-8-oxo-8*H*-pyrano[3,2-*g*]benzoxazole-7-carboxamide (V)——After a mixture of 1 g of IV and 20 ml of $SOCl_2$ had been heated under reflux for 1 hr, the excess of $SOCl_2$ was removed *in vacuo*. The residue was suspended in 30 ml dried benzene and then 20 ml of 5% 2-aminopyridine benzene solution was gradually added to the suepension at below 10°. After standing at room temperature for 24 hr, benzene was evaporated *in vacuo* and the residue was treated with 2% aq. AcOH solution in an ice bath. The resulting solid was collected by suction, washed with H_2O,dried and recrystallized from AcOH to give the product (V) as light yellow needles (1 g), mp>300°. *Anal.* Calcd. for $C_{17}H_{16}O_4N_3$: C, 63.55; H, 3.42; N, 13.08. Found: C, 63.24; H, 3.51; N, 12.87.

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Studies on Peptides. XXIV.[1,2] Some Observation on the Urethan Formation during the Mixed Anhydride Procedure in Peptide Synthesis

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Control of racemization during the mixed anhydride procedure[4–6] in peptide synthesis has been studied by Applewhite, *et al.*[7] and more recently by Anderson, *et al.*,[8,9] especially in the case of acylpeptide anhydrides. Usefulness of this rapid peptide-forming reaction was thus further evaluated. However, the urethane formation, a possible side reaction of this procedure seems to be a limitation in some instances for the use of this method.

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On the theoretical ground, the reaction of the mixed anhydride, *i e.*, a carbonic carboxylic anhydride with an amino component can go in two directions.[10-13] One is the desired direction of the acylpeptide formation and the other is the unfavorable urethan formation. For suppression of such a side reaction, a mixed carbonic carboxylic anhydride with alkyl groups which are electron-releasing part of the molecules is considered to be favorable.[4-6,14-18] For this reason, isobutyl[6,19] or ethyl chloroformate[4,5] is the reagent commonly employed for the peptide synthesis. We wish to report some instances of urethan formation, when such alkyl chloroformates were used in peptide synthesis.

Our first example is concerned with a N-terminal prolyl peptide related to monkey and human β-melanocyte-stimulating hormone.[20,21] Previously we have prepared N^{α}-benzyloxycarbonylprolylprolyl-N^{ε}-formyllysylaspartic acid,[22] a protected tetrapeptide related to the C-terminal portion of the above mentioned MSHs, from N^{α}-benzyloxycarbonylproline and prolyl-N^{ε}-formyllysylaspartic acid by the *p*-nitrophenyl ester method.[23] When this coupling reaction was carried out by means of the mixed anhydride procedure using ethyl or isobutyl chloroformate, the desired protected tetrapeptide could not be isolated in pure form because of the formation of considerable amount of the corresponding urethans. The same compound was also isolated from the reaction of a mixed anhydride of N^{α}-benzyloxycarbonylserylproline with ethyl chloroformate and prolyl-N^{ε}-formyllysylaspatric acid.

It should be considered, as suggested by Buttersby and Robinson,[24] that an urethan might be formed by the direct reaction of amino components and alkyl chloroformates which are not involved in the mixed anhydride formation with a protected amino acid. When a mixture consisting of N^{α}-benzyloxycarbonylproline triethyl ammonium salt and isobutyl chloroformate was poured into a solution of N-hydroxysuccinimide, N^{α}-benzyloxycarbonylproline N-hydroxysuccinimide ester[25] was obtained in excellent yield. From this result, it seems reasonable to assume that the mixed anhydride of N^{α}-benzyloxycarbonylproline in the foregoing experiment proceeded in the normal way and this anhydride went predominantly to the urethan formation. The N-hydroxysuccinimide ester isolated above coupled smoothly, as the corresponding *p*-nitrophenyl ester, with prolyl-N^{ε}-formyllysylaspartic acid to give the protected tetrapeptide mentioned earlier.

It has been reported that secondary amines, including sarcosine tended to react with a mixed anhydride to give urethans.[26] Examples of such urethan formation were reported by Ondetti, *et al.*,[27] Vaughan, *et al.*,[28] and Schnabel, *et al.*[29] where in addition to secondary amines, valyl peptides were used as amino components in this procedure. Considering these results

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and ours, there seems to be an indication that in addition to the nucleophilicity of an amino component,[30] its steric hindrance may also be important in determining the direction of bond formation by the anhydride.

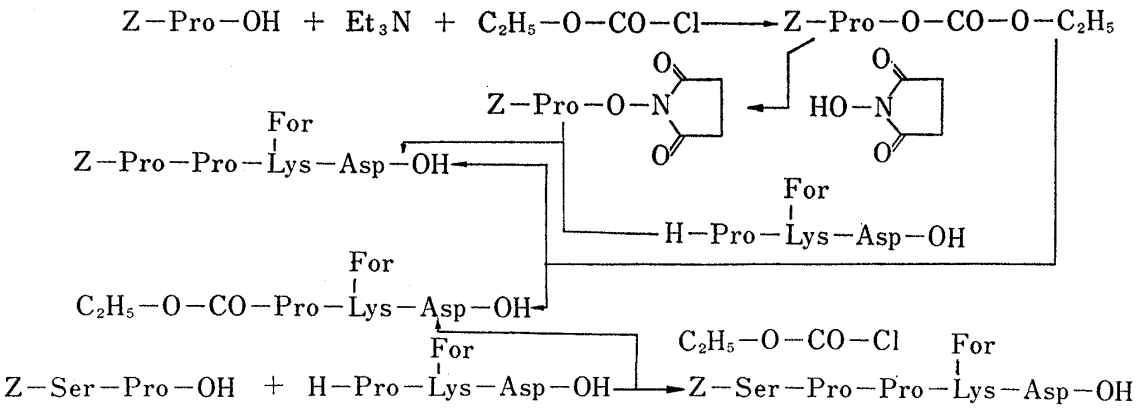

Chart 1

Urethan formation was also briefly mentioned by Vaughan,[31] when relatively large peptides were used as amino component. We have noticed the urethan formation when N^{α}-*t*-butoxycarbonyl-N^{G}-nitroarginine was coupled with the dodecapeptide, methionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysylaspartic acid.[32] The use of active esters was unsuccessful in this instance. When N^{α}-benzyloxycarbonyl-γ-benzylglutamate was condensed with histidylphenylalanylarginyltryptophylglycyl-N^{ε}-formyllysylprolylvaline amide, the formation of an urethan type compound was noted.[33] Such an urethan was also formed when the above octapeptide was substituted with the pentapeptide, histidylphenylalanylarginyltryprophylglycine. After hydrogenation of the reaction product, extensive column chromatography on carboxymethyl cellulose was required to purify the desired hexapeptide, glutamylhistidylphenylalanylarginyltryptophylglycine, from a contaminant of the urethan which exhibited positive colors to Pauly, Sakaguchi and Ehrlich tests but negative to ninhydrin test. An acid hydrolysate of this product contained no glutamic acid. Alternatively, the desired hexapeptide was prepared successfully using the active *p*-nitrophenyl ester of N^{α}-benzyloxycarbonyl-γ-benzylglutamate as reported previously.[34]

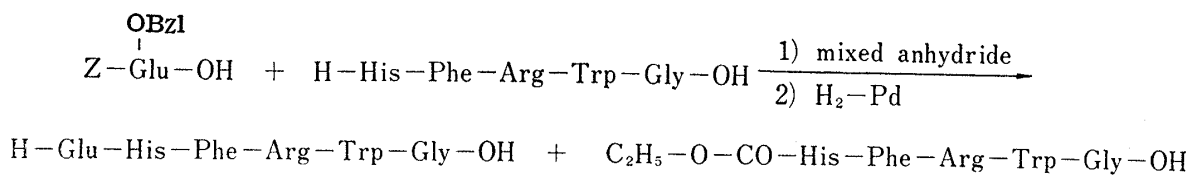

Chart 2

Recently Stewart[35] made a statement that the urethan formation occurs to a relatively small extent in every case in the mixed anhydride reaction and with relatively large peptides, presumably such as penta or hexapeptides, the solubility of both products and urethans tends to be similar and purification difficulties arise. The above experiments may be an example to support his view.

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Generally small peptides prepared by the mixed anhydride method are highly pure since by-products, such as an alcohol, carbon dioxide and an urethan formed in some extent are all easily removable. Indeed this is generally the case in peptide synthesis. In spite of such superior properties of this method and even though risk of racemization has been greately reduced, the urethan formation is certainly one of the limitation of this rapid amide-forming reaction in some instances as recorded.

Experimental

General experimental methods employed are essentially the same as described in the Part XXII[32] of this series. On paper chromatography, *Rf* values refer to the system of *n*-BuOH, AcOH and H_2O (5:1:4). NMR spectra were measured at 60 Mc on a Varian associate A-60 spectrometer.

N^{α}-Ethoxycarbonylprolyl-N^{ε}-formyllysylaspartic Acid——a) A mixed anhydride, prepared from N^{α}-benzyloxycarbonylserylproline (1.04 g) in dioxane (7.5 ml) with tri-*n*-butylamine (0.74 ml) and ethyl chloroformate (0.3 ml), was added to a solution of prolyl-N^{ε}-formyllysylasparitc acid (1.20g) and triethylamine (0.86 ml) in 60% aqueous dioxane (10 ml). The mixture was stirred in an ice-bath for 30 min and then at room temperature for 2 hr. The most of the solvent was evaporated and the pH of the residue was adjusted to 4 with 1N HCl. The solution was kept in a refrigerator overnight to form a solid, which was separated by filtration (see below for trituration of the mother liquid), washed with a small amount of 10% citric adid, dried over P_2O_5 and KOH pellets *in vacuo* and recrystallized from MeOH and AcOEt; yield 0.86 g (61%), mp 195—198°. $[\alpha]_D^{22}$ −57.0° (c=0.9, MeOH). The product is ninhydrin negative and soluble in H_2O. Amino acid ratios in an acid hydrolysate $Pro_{1.04}Lys_{0.91}Asp_{1.00}$ (average recovery 91%). NMR spectra, τ=5.88 quartet, J=7 cps and τ=8.80, triplet, J=7 cps. No carbon dioxide evolved by hydrogenolysis. *Anal.* Calcd. for $C_{19}H_{30}O_9N_4$: C, 49.8; H, 6.6; N, 12.2. Found: C, 49.6; H, 6.8; N, 11.9.

The mother liquid was extracted with *n*-BuOH, which was evaporated to give a semisolid (0.05 g). This crude product was submitted to acid hydrolysis; $Ser_{0.40}Pro_{1.34}Lys_{0.94}Asp_{1.00}$. The result indicated that very little N^{α}-benzyloxycarbonylserylprolylprolyl-N^{ε}-formyllysylaspartic acid which still contaminated with the above urethan formed in this reaction.

b) A mixed anhydride, prepared from N^{α}-benzyloxycarbonylproline (0.36 g) in dry tetrahydrofuran (3 ml) with tri-*n*-butylamine (0.37 ml) and ethyl chloroformate (0.14 ml) was added to a solution of prolyl-N^{ε}-formyllysylaspartic acid (0.60 g) and triethylamine (0.4 ml) in 70% aqueous dioxane (9 ml). Isolation of the urethan was performed as stated above; yield 0.36 g (55%), mp 196—198°. Identity of this product with the urethan obtained in (a) was established by comparison of their IR spectra and mixed melting point.

The filtrate of the above product was extracted with *n*-BuOH, which after washing with H_2O, was evaporated to give a powder (0.21 g). This crude product was hydrogenated over a Pd catalyst in MeOH. After evaporation of the solvent, the residue was extracted with *n*-BuOH to remove the last trace of the urethan and the aqueous layer was separated and lyophilized to give a ninhydrin positive powder, 0.12 g (16%), *Rf* 0.13. This product was identical with the tetrapeptide, prolylprolyl-N^{ε}-formyllysylaspartic acid prepared by the alternate method.[22] From this result, the ratio of the tetrapeptide and the urethan can be judged as approximately 1 to 4.

N^{α}-Isobutyloxycarbonylprolyl-N^{ε}-formyllysylaspartic Acid——Ethyl chloroformate in the above experiment (b) was substituted with isobutyl chloroformate (0.19 ml). Reaction and subsequent isolation were carried out as described above to obtain the corresponding urethans; yield 0.30 g (43%), mp 182—184°, $[\alpha]_D^{30}$ −48.0° (c=1.0, MeOH). *Anal.* Calcd. for $C_{21}H_{34}O_9N_4$: C, 51.8; H, 7.0; N, 11.5. Found: C, 51.7; H, 7.0; N, 11.3.

N^{α}-Benzyloxycarbonylprolyl-N-hydroxysuccinimide Ester——Isobutyl chloroformate (2.6 ml) was added to an ice-cooled solution of N^{α}-benzyloxycarbonylproline (4.98 g) and triethylamine (1.4 ml) in dry tetrahydrofuran (30 ml). This solution, after stirring for 30 min, was added to a solution of N-hydroxysuccinimide (2.02 g) in dry tetrahydrofuran (10 ml). The mixture was stirred in an ice-bath for 30 min and at room temperature for 2.5 hr. After filtration, the solvent was evaporated and the residue was treated with ether to give a powder, which was recrystallized from isopropyl alcohol; yield 4.85 g (73%), mp 89—91° (lit.[25] mp 90°). The product and the product obtained by the dicyclohexylcarbodiimide procedure according to Anderson, *et al.*[25] were identical in mixed mp and IR spectra. *Anal.* Calcd. for $C_{17}H_{18}O_6N_2$: C, 59.0; H, 5.2; N, 8.1. Found: C, 59.0; H, 5.4; N, 8.3.

Reaction of N^{α}-Benzyloxycarbonylproline-N-hydroxysuccinimide Ester with Prolyl-N^{ε}-formyllysylaspartic Acid——Prolyl-N^{ε}-formyllysylaspartic acid (0.15 g) was dissolved in an aqueous solution (4 ml) of sodium bicarbonate (0.07 g) and a solution of N^{α}-benzyloxycarbonylproline-N-hydroxysuccinimide ester (0.17 g)in EtOH (4 ml) was combined. The soluion was stirred at room temperature for 48 hr. The solvent was evaporated and the residue was dissolved in H_2O, which was washed with AcOEt and then condensed to a small volume. The resulting solution was acidified with 1N HCl and was extracted with *n*-BuOH, which

after washing with H_2O, was evaporated to dryness. Addition of AcOEt to the residue formed a solid, which was recrystallized from MeOH and AcOEt; yield 0.16 g (71%). $[\alpha]_D^{22}$ −67.0° (c=1.0, MeOH). Identity of this product and the authentic sample of N^{α}-benzyloxycarbonylprolylprolyl-N^{ε}-formyllysylaspartic acid[22] was established by comparison of their *Rf* values (both 0.53, by the iodine stain) and their IR spectra.

Reaction of a Mixed Anhydride of N^{α}-Benzyloxycarbonyl-γ-benzylglutamate with Histidylphenylalanayl-arginyl Tryptophylglycine——A mixed anhydride, prepared from N^{α}-benzyloxycarbonyl-γ-benzylglutamate (0.22 g) in tetrahydrofuran (6 ml) with triethylamine (0.08 ml) and ethyl chloroformate (0.07 ml) was added to a solution of histidylphenylalanylarginyltryptophylglycine acetate[32] (0.17 g) and triethylamine (0.03 ml) in dimethylformamide (6 ml) and the solution was stirred in an ice–bath for 3 hr. The solvent was evaporated and the residue was treated with AcOEt. The resulting powder was washed successively with AcOEt and H_2O; yield 0.15 g. *Rf* 0.77 and 0.53; both ninhydrin negative but Pauly, Sakaguchi and Ehrlich positive spots. This powder in 20% AcOH (25 ml) was hydrogenated over a Pd catalyst at room temperature for 7 hr. During the hydrogenation, the spot of *Rf* 0.53 remained unchanged while the spot of *Rf* 0.77 disappeared and new ninhydrin positive spot of *Rf* 0.33 was detected. The catalyst was removed by filtration and the filtrate was evaporated. The residue in H_2O (150 ml) was applied to a column of CM-cellulose (2×13 cm), which was eluted with 0.05M pyridine acetate buffer at pH 5.0. Absorbancy at 280 mμ was determined in each fraction (20 ml each). Two peaks were detected and the solvents of each peak were evaporated to dryness and the residues were lyophilized respectively; yield of the front peak (tube No. 55 to 75), 0.023 g (12%), $[\alpha]_D^{20}$ −21.0° (c=0.5, 1N AcOH), *Rf* 0.53 ninhydrin negative but Pauly, Sakaguchi and Ehrlich positive spot. Amino acid ratios in an acid hydrolyaste $His_{0.97}Phe_{1.00}Arg_{0.96}Gly_{1.05}$ (average recovery 85%). Yield of the behind peak (tube No. 90 to 118), 0.065 g (32%), $[\alpha]_D^{20}$ −13.5° (c=0.6, 1N AcOH), (c=lit.[36] $[\alpha]_D$ −15° in 1N AcOH, lit.[37] $[\alpha]_D^{25}$ −17.3° in AcOH, lit.[38] −18.0° in 1N AcOH). *Rf* 0.33, single spot positive to ninhydrin, Pauly, Sakaguchi and Ehrlich test. Amino acid ratios in an acid hydrolysate $Glu_{1.07}His_{1.11}Phe_{0.94}Arg_{1.00}Gly_{0.99}$ (average recovery 86%).

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Studies on Peptides. XXV.[1] A Convenient Procedure for the Preparation of *p*-Methoxybenzyl Azidoformate

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Recently we described a convenient procedure for the preparation of *tert*-butyl azidoformate, which involved the direct reaction of *tert*-butyl chloroformate and hydrazoic acid in the presence of a base.[3] We have now found that this procedure, with slight modification,

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