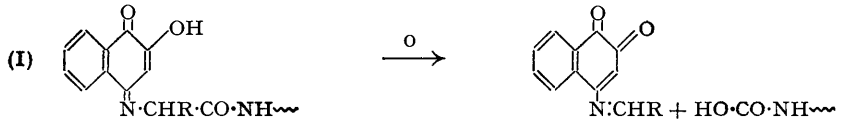
386. *Peptides. Part II.* Selective Degradation by Removal of the Terminal Amino-acid bearing a Free Amino-group. The Use of Alkyl Alkoxydithioformates (Dialkyl Xanthates).*

By G. W. KENNER and H. G. KHORANA.

Peptides are converted by the action of dialkyl xanthates on their sodium salts in aqueous solution at room temperature into their *N*-thioncarbalkoxy-derivatives (V). These substances are cleaved by hydrogen chloride in nitromethane to the hydrochloride of an amino-acid or degraded peptide and a 4-alkylthiazolid-2 : 5-dione (VIII), from which the terminal amino-acid may be regenerated by mild hydrolysis. The two steps proceed in almost quantitative yield and in combination constitute a valuable method for selective degradation of peptides.

THE structure of peptides has been determined by partial hydrolysis with acids or enzymes and identification of the various di- and tri-peptides liberated (*e.g.*, Consden, Gordon, Martin, and Synge, *Biochem. J.*, 1947, **41**, 596; Šorm and Keil, *Chem. Listy*, 1951, **45**, 278). In combination with partial hydrolysis of the dinitrophenyl derivative of the original substance (Sanger, *Biochem. J.*, 1945, **39**, 507) this is a very powerful method of analysis, as has been demonstrated by Sanger and Tuppy in their elegant work on insulin (*ibid.*, 1951, **49**, 463, 481). Nonetheless the solution of future problems would be assisted by methods of selective degradation, *i.e.*, by cleavage of a peptide link specified either by its function in binding a particular amino-acid or by its proximity to an end of a chain. The latter type of method has attracted several investigators (cf. Fox, *Adv. Protein Chem.*, 1945, **2**, 155) and this paper is concerned with our own studies of degradation of a peptide chain from the end bearing a free amino-group.

Selective removal of the terminal amino-acid involves two stages : attack by a reagent which either modifies the terminal amino-acyl group so as to reduce its stability or attaches a residue so constructed that its reaction with the neighbouring peptide linkage is facilitated by formation of a stable cyclic structure; the amino-acid at one remove from the end is then uncovered in the second step. A method of the former class described by Goldschmidt and his colleagues (Goldschmidt, Wiberg, Nagel, and Martin, *Annalen*, 1927, **456**, 1; Goldschmidt and Strauss, *ibid.*, 1929, **471**, 1) depends on elimination of the terminal amino-acid as a nitrile by action of alkaline hypobromite, and has various practical disadvantages. Our first experiments were directed along somewhat similar lines. Like ninhydrin, sodium

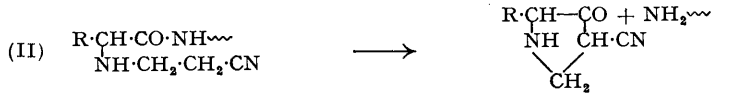


1 : 2-naphthaquinone-4-sulphonate reacts with amino-acids liberating carbon dioxide (Van Slyke, Dillon, MacFadyen, and Hamilton, *J. Biol. Chem.*, 1941, **141**, 627), and it also reacts with peptides (Frame, Russell, and Wilhelmi, *ibid.*, 1943, **149**, 255) presumably

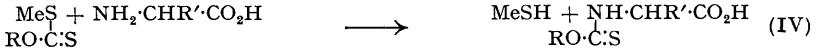
* Part I, preceding paper.

giving compounds such as (I). We envisaged the possibility that these might be oxidised according to the scheme shown above, but all attempts with silver oxide, hydrogen peroxide, sodium bismuthate, and lead tetra-acetate were unsuccessful. The colour was destroyed, but with production of the original and not the degraded peptide.

The second type of approach, elimination of the terminal amino-acid in a cyclic structure, has in the past been more fruitful. Edman (*Acta Chem. Scand.*, 1950, **4**, 283) has developed a method of considerable promise by substituting phenyl *iso*thiocyanate for the phenyl *iso*cyanate used by earlier workers (Bergmann, Miekeley, and Kann, *Annalen*, 1927, **458**, 56; Abderhalden and Brockmann, *Biochem. Z.*, 1930, **225**, 386); a prominent feature of his process is that the second step is acid-catalysed cyclisation in an anhydrous medium of the phenylthiocarbamyl derivative initially formed in aqueous solution. Levy (*J.*, 1950, 404)



has shown that 4-alkyl-2-thiothiazolid-5-ones are split off when salts of dithiocarboxy-peptides are acidified and has utilised this reaction in a method of degradation in aqueous solution throughout. Among various possible reaction sequences explored by us was addition of the peptide to acrylonitrile (cf. McKinney, Uhing, Setzhorn, and Cowan, *J. Amer. Chem. Soc.*, 1950, **72**, 2599) and fission of the product (II) to a 2-alkyl-4-cyanopyrrolid-3-one. (*N*-2-cyanoethyl-DL-alanyl)glycine was obtained crystalline in satisfactory yield and did give some glycine on treatment with alkali; the reaction was however sluggish and could not be brought to completion without the use of drastic conditions, and the compound was not affected by dilute acid. Another attractive possibility was to thioformylate the peptide with sodium dithioformate in aqueous solution (Todd, Bergel, and



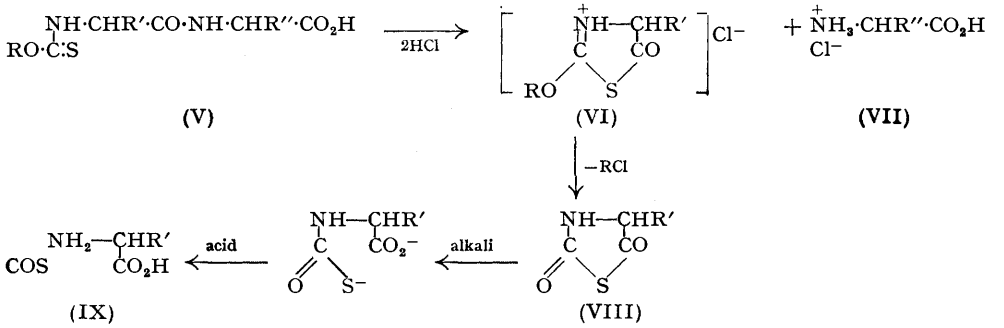
Karimullah, *J.*, 1936, 1557) and then to eliminate the terminal amino-acid as a 4-alkylthiazol-5-one. But attempts to thioformylate amino-acids were unsuccessful. The difficulty could have been overcome by simply replacing this salt by the ester of another dithio-acid (McOmie, *Ann. Reports*, 1948, **45**, 209) and indeed Kjaer (*Acta Chem. Scand.*, 1950, **4**, 1347) has recently accomplished the satisfactory thiobenzoylation of amino-acids. However at this time Aubert and Knott (*Nature*, 1950, **166**, 1039; Aubert, Knott, and Williams, *J.*, 1951, 2185) described the reaction between dialkyl xanthates and glycine, yielding *N*-thioncarbalkoxyglycines (IV; R′ = H). By use of this observation one of us (Khorana, *Chem. and Ind.*, 1951, 129) has been able to develop a new practical method of peptide degradation.

The *N*-thioncarbalkoxyglycines (IV; R′ = H) were obtained in fair yield by Aubert, Knott, and Williams (*loc. cit.*) by boiling aqueous solutions of the potassium salt of glycine during 18 hours with the ethyl xanthates. These conditions are unnecessarily vigorous: when the methyl esters (III) are used, methanethiol is evolved at room temperature from the homogeneous reaction mixture in aqueous 2-ethoxyethanol. Extraction by ethyl acetate in presence of acetic acid, after removal of excess of reagent with ether, affords the desired products in almost quantitative yield. The generality of the reaction was demonstrated by the preparation in high yield of crystalline derivatives (IV; R = Et) of glycine, DL-alanine, and DL-valine. Even the imino-group of DL-proline was acylated without difficulty. Similarly the dipeptides, glycylglycine, glycyl-DL-valine, glycyl-DL-leucine, glycyl-DL-phenylalanine, DL-alanylglycine, and DL-leucylglycine all gave crystalline *N*-thioncarbethoxy-derivatives (V; R = Et) in high yield.

In these compounds (V) the sulphur atom is favourably placed for reaction with the neighbouring peptide link as in the scheme shown above. Accordingly the derivative of DL-alanylglycine (V; R = Et, R′ = Me, R″ = H) was subjected to the same treatment as in Edman's method of degradation (*loc. cit.*); hydrogen chloride was bubbled through its solution in nitromethane with rigorous exclusion of moisture. As expected, glycine

hydrochloride separated almost immediately and was collected after a few hours in very good yield. The amino-acid hydrochlorides (VII) were likewise obtained from all the other *N*-thioncarbethoxy-dipeptides (V; R = Et).

The nitromethane liquors contained the other amino-acid residue (IX) bound in a cyclic structure. Aubert and Knott (*loc. cit.*; Aubert, Jeffreys, and Knott, *J.*, 1951, 2195) showed that *N*-thioncarbethoxyglycine (IV; R = Et, R′ = H) is easily converted by phosphorus trichloride in benzene into thiazolid-2 : 5-dione (VIII; R = H). The same substance was produced by the action of phosphorus tribromide in moist benzene on 2-ethoxythiazol-5-one, the free base of (VI; R = Et, R′ = H). We therefore presume that (VIII) represents the cyclic end-products of our degradation, although their instability has precluded their actual isolation. The syrups, remaining after evaporation of the nitromethane, are converted by brief treatment with dilute alkali and then acid into the free terminal amino-acid (IX), identified in our experiments by paper chromatography. Omission of the first step in hydrolysis leads to polypeptides in addition to the amino-acid itself (cf. Aubert, Jeffreys, and Knott, *loc. cit.*).



These experiments with dipeptides established the general form of the new technique of selective degradation. It was equally successful with the tripeptide DL-leucylglycylglycine, but its application to glycylglycine and to glycylglycylglycine was a little troublesome. In these cases the *N*-thioncarbethoxy-derivatives were sparingly soluble in cold nitromethane and, when the degradation step was carried out in warm solution, the recovered terminal glycine was contaminated with polypeptides. These are clearly exceptional cases and, should similar difficulties arise with substances of greater importance, they could probably be overcome by change to a more polar inert solvent. However, as *N*-thioncarbohexyloxyglycine (IV; R = *n*-C_6H_{13}) is soluble in light petroleum (Aubert, Knott, and Williams, *loc. cit.*), it was of interest to examine the effect of enlarging the alkyloxy-group. The intermediates such as (V; R = Bu^n, R′ = R″ = H), prepared from *n*-butyl methyl xanthate, were indeed more soluble, but the undesirable features were not completely removed by this small increase in chain length.

The degraded peptide need not be collected as its crystalline hydrochloride, but may be extracted from the nitromethane solution by water (see Experimental). Addition of the appropriate amount of alkali to the aqueous solution then gives directly the sodium salt of the degraded peptide ready for reaction with a second portion of the methyl xanthate.

Successive applications of the method could, in principle, lead to complete elucidation of the sequence of amino-acids in a peptide chain, and it should therefore be useful for structural determination in natural products. For this purpose it appears to offer some advantages over those described by Levy (*loc. cit.*) and Edman (*loc. cit.*). In the former the two steps are carried out consecutively in the same aqueous solution and there is thus a risk of contamination of the degraded with the original peptide. This is completely avoided in our method by extraction of the intermediates, which are stable to aqueous acid, into an organic solvent from an acid aqueous medium. In Edman's method, on the other hand, the initial reaction with phenyl *iso*thiocyanate has to be carried out under carefully controlled conditions and the isolation of the phenylthiocarbamyl-peptides is more troublesome; the solubilities of these compounds are also less than those of the *N*-thioncarbalkoxy-derivatives.

Experimental

M. p.s are uncorrected.

Preparation of Alkyl Methyl Xanthates.—Potassium ethyl and butyl xanthate were prepared according to Vogel's directions (*J.*, 1948, 1833). Methyl iodide in methyl alcohol (the vigorous reaction was moderated at first by external cooling) under reflux for ½ hour afforded *O*-ethyl (III; R = Et) and O-n-*butyl methyl xanthate* (III; R = Bu^n), b. p. 60°/0·5 mm., as a light yellow liquid (Found: C, 44·1; H, 7·0. $C_6H_{12}OS_2$ requires C, 43·9; H, 7·4%).

General Method of Preparation of N-*Thioncarbalkoxy-derivatives of Amino-acids and Peptides.*—The amino-acid or the peptide is dissolved in 5N-sodium hydroxide (*ca.* 1·1 mol.) and approximately 4 mol. of the dialkyl xanthate added. The contents are then homogenised by the dropwise addition, with shaking, of 2-ethoxyethanol (3—4 parts to 1 part of the aqueous alkaline solution). Methanethiol begins to be evolved soon and the reaction is completed by keeping the clear solution at room temperature for 24—48 hours. The contents are then evaporated *in vacuo* to a syrup, diluted with water, and extracted with ether to remove excess of the reagent. The *N*-thioncarbalkoxy-derivative is then isolated by repeated extraction with ethyl acetate in presence of acetic acid. In the case of amino-acids, the acidification may be carried out with hydrochloric acid.

The compounds prepared by the general method (illustrated below in detail for one case) in quantitative yield are listed in the table. DL-Leucine and DL-phenylalanine gave oils.

				Found, %:			Required, %		
Amino-acid	M. p.	Solvent for crystn.†	Formula	C	H	N	C	H	N
* Glycine	98—99°	Et_2O–Pet	$C_5H_9O_3NS$	36·8	5·2	—	36·8	5·6	—
* DL-Alanine	102	C_6H_6–Pet	$C_6H_{11}O_3NS$	40·6	6·0	8·0	40·7	6·3	7·9
DL-Valine.....	98	C_6H_6–Pet (sublimed 80°/10^{-3} mm.)	$C_8H_{15}O_3NS$	47·1	7·2	6·7	46·8	7·4	6·8
DL-Proline ...	128—129	C_6H_6–Pet	$C_8H_{13}O_3NS$	47·6	6·5	6·8	47·3	6·4	6·9

* See also Aubert, Knott, and Williams (*loc. cit.*). † Pet = light petroleum (b. p. 40—60°).

N-*Thioncarbethoxy*-DL-*alanylglycine* (V; R = Et, R′ = Me, R″ = H).—To a solution of DL-alanylglycine (0·146 g.) in 5N-sodium hydroxide (0·22 c.c., 1·1 equivalents) *O*-ethyl methyl xanthate (0·52 g., *ca.* 4 mol.) was added followed by 2-ethoxyethanol (*ca.* 1 c.c.) till a clear light yellow solution resulted. After 48 hours at 18—20°, the contents were evaporated to a thick syrup *in vacuo* and diluted with water (5 c.c.). The excess of reagent was removed by 3 extractions with ether; the combined ethereal extracts were washed with a small volume of water, and the washings added to the main bulk of the aqueous solution. After addition of acetic acid (1 c.c.) the solution was extracted twice with ethyl acetate; this was followed by the addition of a further 0·5 c.c. of the acid and continued extraction. In all, six extractions (volume of combined extracts, *ca.* 50 c.c.) were carried out after the portionwise addition of 2 c.c. of acetic acid. After being dried ($CaSO_4$) the extracts were evaporated under partial vacuum, finally in a high vacuum to remove last traces of acetic acid. N-*Thioncarbethoxy*-DL-*alanylglycine* (0·198 g., 86%) crystallised directly after prolonged suction. A sample, recrystallised from nitromethane and washed with small volume of diethyl ether, had m.p. 143—144° (Found, after drying for 18 hours at 80°: C, 41·4; H, 5·8; N, 11·9; $C_8H_{14}O_4N_2S$ requires C, 41·0; H, 6·0; N, 12·0%).

Following the general method of cleavage described below, the compound was degraded in anhydrous nitromethane to give almost immediately glycine hydrochloride in very good yield (over 70% directly) and the substituted thiazolid-2 : 5-dione containing the terminal DL-alanine residue. The latter was recovered by the method outlined below, and no polymerisation products were detected.

General Method of Cleavage of N-*Thioncarbalkoxy-peptides.*—The dried *N*-thioncarbalkoxy-peptide is dissolved in anhydrous nitromethane (by gentle warming, if necessary), and the solution is saturated with dry hydrogen chloride under complete exclusion of moisture. The hydrochloride of the degraded amino-acid or peptide sometimes begins to separate immediately. In every case the crystalline product is collected after a few hours and washed with small amounts of dry ether. The clear nitromethane solution is evaporated carefully at low temperature and the residue treated with N-sodium hydroxide and neutralised with hydrochloric acid after 5 minutes. The amino-acid thus liberated is identified by paper chromatography. Sometimes small amounts of polypeptides are also detected on the paper chromatogram.

In small-scale work a convenient modification consists in addition of water to the anhydrous nitromethane solution after completion of the degradation. The aqueous layer, after separation, is washed with a small volume of ether, and the organic solvent layer similarly with a small

amount of water. The degraded amino-acid or peptide and the terminal amino-acid are then easily recovered from the aqueous solution and the organic solvent respectively.

N-*Thioncarbethoxy*-DL-*leucylglycine* (V; R = Et, R′ = Bu^i, R″ = H) was prepared, according to the general method, in practically quantitative yield as an oil which slowly crystallised. Recrystallised from ether–light petroleum (b. p. 40—60°) and finally from water, it had m. p. 122° (Found: C, 47·9; H, 6·9; N, 10·3. $C_{11}H_{20}O_4N_2S$ requires C, 47·8; H, 7·3; N, 10·1%).

The degradation was carried out according to the general procedure. Glycine hydrochloride separated immediately in good yield and formed long needles. DL-Leucine was recovered from the " cleavage product " and was found, by paper chromatography, to be free from any polypeptides.

N-*Thioncarbethoxyglycylglycine* (V; R = Et, R′ = R″ = H) was prepared as above, 24 hours being sufficient for the completion of the reaction (yield in two experiments, 187 and 196 mg.; theor., 220 mg.). Recrystallisation from nitromethane afforded clusters of needles, m. p. 142—143° (Found, in a sample dried at 80° for 18 hours: C, 38·4; H, 5·2; N, 12·2. $C_7H_{12}O_4N_2S$ requires C, 38·2; H, 5·5; N, 12·7%).

Degradation of this *compound* was not completely satisfactory as it was necessary to employ a warm nitromethane solution of the substance. The terminal glycine residue as recovered from the nitromethane solution was, in this case, contaminated with some slow-moving polypeptides.

N-*Thioncarbethoxyglycyl*-DL-*valine* (V; R = Et, R′ = H, R″ = Pr^i) was obtained in theoretical yield as an oil which gradually crystallised. Recrystallised from ether–light petroleum (b. p. 40—60°) and then from water it separated as rosettes of needles, m. p. 131—132° (Found, in a sample dried at room temperature; C, 45·9; H, 6·9; N, 10·5. $C_{10}H_{18}O_4N_2S$ requires C, 45·8; H, 6·9; N, 10·7%).

The clear nitromethane solution saturated with hydrogen chloride deposited DL-valine hydrochloride within ½ hour at room temperature.

N-*Thioncarbethoxyglycyl*-DL-*leucine* (V; R = Et, R′ = H, R″ = Bu^i), obtained as an oil in the usual way and dried in high vacuum, afforded crystals (84%) on dissolution in benzene–light petroleum (b.p. 40—60°). One recrystallisation from water gave spherical clusters of the needles, m. p. 109—110°; a different sample melted partly at 110° and then completely at 120° (Found: C, 48·2; H, 7·3; N, 10·1. $C_{11}H_{20}O_4N_2S$ requires C, 47·8; H, 7·3; N, 10·1%).

On being kept for a few hours, a clear cold nitromethane solution, saturated with hydrogen chloride, deposited long needles of DL-leucine hydrochloride in practically quantative yield.

N-*Thioncarbethoxyglycyl*-DL-*Phenylalanine* (V; R = Et, R′ = H, R″ = CH_2Ph) was obtained in quantitative yield as an oil which crystallised from aqueous acetone and recrystallised from water as clusters of glistening needles, m. p. 74—75° (Found, after drying in air at room temperature: C, 51·0; H, 5·8; N, 8·3. $C_{14}H_{18}O_4N_2S,1H_2O$ requires C, 51·2; H, 6·1; N, 8·5%).

The *compound* underwent, as above, ready degradation in nitromethane solution.

N-*Thioncarbethoxy*-DL-*leucylglycylglycine*, obtained as an oil in 93% yield, afforded crystalline material from ether–light petroleum (b. p. 40—60°). Recrystallisation from nitromethane gave clusters of fine needles, m. p. 126—127° (Found: C, 46·7; H, 6·6; N, 12·4. $C_{13}H_{23}O_5N_3S$ requires C, 46·8; H, 7·0; N, 12·6).

When hydrogen chloride was bubbled through a clear nitromethane solution of the *compound*, glycylglycine hydrochloride soon appeared first as an oil which solidified.

N-*Thioncarbo*-n-*butyloxyglycylglycine* (V; R = Bu^n, R′ = R″ = H) was prepared as above from glycylglycine (0·132 g.) and *O*-*n*-butyl methyl xanthate (0·70 g.) in the presence of 5N-sodium hydroxide (0·22 c.c., 1·1 mol.). Recrystallised from ether–light petroleum (b. p. 40—60°) it had m. p. 84—85° (Found: C, 43·6; H, 6·5; N, 11·2. $C_9H_{16}O_4N_2S$ requires C, 43·5; H, 6·5; N, 11·3%).

The degradation of this *compound* was carried out as in the case of the corresponding *N*-thioncarbethoxy-derivative in warm nitromethane solution and the terminal glycine after recovery showed contamination with polypeptides.

N-*Thioncarbo*-n-*butyloxyglycylglycylglycine*, prepared in 82% yield as above and recrystallised from nitromethane, had m. p. 152—153° (Found: C, 43·3; H, 6·5; N, 13·9. $C_{11}H_{19}O_5N_3S$ requires C, 43·3; H, 6·3; N, 13·8%).

The degradation was carried out as for the foregoing compound in warm nitromethane solution and was accompanied by the formation of slight colour.

Reaction of DL-*Alanylglycine with Sodium* 1 : 2-*Naphthaquinone*-4-*sulphonate and Attempted Oxidative Degradation.*—To a solution of DL-alanylglycine (0·146 g.) in N-sodium hydroxide (1 c.c.) was added sodium 1 : 2-naphthaquinone-4-sulphonate (0·26 g.), and the intense red

solution thus obtained was kept for 24 hours. The red product was extracted with *n*-butanol after acidification of the contents with hydrochloric acid. An aqueous solution of the red substance was shaken with freshly precipitated silver oxide, the red colour disappearing almost completely. Similar treatment with dilute hydrogen peroxide followed by addition of silver oxide to remove excess of hydrogen peroxide also caused the disappearance of the colour. The aqueous solutions were examined by paper chromatography and DL-alanylglycine and traces of glycine were found to be present. The oxidation in water and aqueous acetic acid with sodium perbismuthate and lead tetra-acetate in glacial acetic acid also gave rather similar results.

Attempted Reaction of Sodium Dithioformate with Amino-acids.—An aqueous solution of sodium dithioformate (1·24 g.) and DL-alanine (0·445 g.) in the presence of an equivalent amount of sodium hydroxide was kept for 48 hours. No appreciable reaction occurred and the major product isolated was a polymer of dithioformic acid. Varying the conditions of pH did not materially alter this result. Similarly, no reaction was observed between DL-phenylalanine or glycine and the above mentioned reagent.

N-2-*Cyanoethyl*-DL-*alanylglycine and its Attempted Degradation.*—A mixture of an aqueous solution of DL-alanylglycine (0·292 mg.) containing sodium hydroxide (N; 2 c.c.) and acrylonitrile (0·12 g.) was shaken for 24 hours; the oily layer disappeared almost completely. After one extraction with ether the clear solution was acidified to Congo-red with hydrochloric acid and concentrated *in vacuo*. Addition of ethanol followed by cooling afforded N-2-*cyanoethyl*-DL-*alanylglycine* as a mass of fine needles in 70% yield. Two further recrystallisations from hot ethanol gave material with constant m. p. (150°) (Found : C, 48·2; H, 6·6; N, 21·2. $C_8H_{13}O_3N_3$ requires C, 48·2; H, 6·6; N, 21·1%).

An aqueous solution of the foregoing compound was stable in dilute hydrochloric acid solution. Treatment of the substance with N-alkali at room temperature, and sodium methoxide in boiling toluene resulted in the formation of glycine among some other products which were detected on a paper chromatogram (phenol : water). Higher concentrations of alkali caused the degradation of DL-alanylglycine itself. A solution of the cyanoethyl derivative in dimethylformamide was stable in the presence of triethylamine.

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