CYANOMETHYLDITHIOBENZOATE AS A NEW REAGENT FOR THE SEQUENTIAL DEGRADATION OF PEPTIDES.

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SUMMARY - The synthesis and purification of cyanomethyldithiobenzoate is described. This active dithio ester can be used for degrading peptides, stepwise from their amino end, under mild conditions.

The Edman reaction (1) is the one which is used mostly today for the determination of amino acid sequences, and has been adapted to the automatic degradation of proteins (2) and peptides (3). It appears, however, that there are limitations to the use of the reaction for a very large number of stages, probably because of side reactions which are liable to occur under the action of the trifluoroacetic or heptafluorobutyric acids used during the cyclization step repeated at each stage. These reagents, indeed, tend to promote spurious hydrolysis of non-terminal peptide bonds in the chain, especially at the level of some residues (4). It therefore appeared that if a sequential degradation of peptides was to be useful for very extended operations, a reagent ought to be found which could give rise to a derivative cyclizing under less drastic conditions that those (40°C, 20 min., or 50°C, 6-7 min.) which are prevalent with Edman's reagent (5, 2, 6).
A consideration of the mechanism of the reaction of this step :

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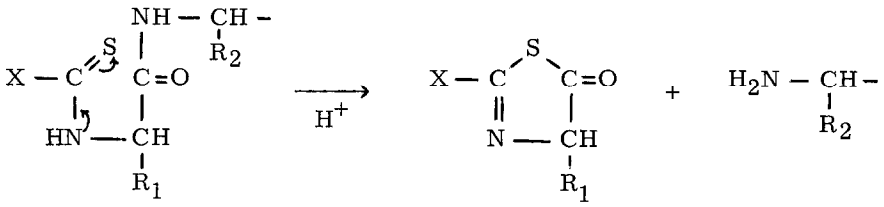

suggested that such a purpose would be achieved if the sulfur atom in the thiocarbamyl group could be made more nucleophilic by a less electron withdrawing X substituent than is the case with the anilino residue. This prediction was indeed substantiated independently by the work of Barrett on thiobenzoylpeptides (7) which appeared shortly after the present work was initiated. Nevertheless a reagent which could condensate with free amino-groups to yield the appropriate thioacylderivative, under conditions which would be as favourable as those used with Edman's phenylisothiocyanate (PITC), was not available.

Previous work from this laboratory (4) indicated that thioacid chlorides, and especially thiopivaloyl - and thiobenzoyl chlorides, would be highly appropriate. However, their instability precluded their synthesis. The potential usefulness of several active dithio esters was therefore explored, as their oxygen equivalents have proven so useful in peptide synthesis (8, 9). In the present communication, such an exploration is described as well as the promising results obtained with the cyanomethyl ester of dithiobenzoic acid (CMDTB).

Synthesis of dithiobenzoic acid esters.

All the esters were obtained by condensation of the sodium salt of dithiobenzoic acid (10) with the appropriate chloro derivative. The carboxymethyl- (10), methoxymethyl- and cyanomethyl dithiobenzoates could thus be readily obtained, generally as crystalline solids. As, however, kinetic considerations restricted the use of the first two esters for the present purpose, only the synthesis of the last one will be described in detail, as an example.

To 0.1 mole sodium dithiobenzoate in 135 ml H_2O, 0.09 mole chloracetonitrile (6 ml, Fluka) were added, and the mixture was gently stirred magnetically overnight at room temperature. The crude ester, which precipitated during this time as dark red droplets, was collected by decantation and washed twice with distilled water. This crude ester was purified on a

column of silicagel (Prolabo n° 24.355) in benzene, and the pure effluent product, which was easily identified by its distinct cherry-red colour, was crystallized from ether-heptane after evaporation of the solvent. Yield : 3.68 g.; m.p. (uncorrected) : 38-41°C; 55.2 % C, 7.2 % N, 3.7 % H (theoret. : 56.0, 7.2, 3.6, respectively); ε = 15.4 x 10^3 liter $mole^{-1}$ cm^{-1} at 305 mμ in benzene. Skin irritant !

Condensation of dithiobenzoates with amino acids and peptides.

This step was examined by reacting ca. 100 μ moles ester with 6 μ moles dipeptide in a total volume of 0.125 ml aqueous buffers containing 40-60 % pyridine, spotting 10 λ aliquots of the reaction mixture at various times on a sheet of Whatman # 3 MM paper, developping the chromatogram overnight with n-butanol : acetic acid : water (7:1:2, one phase) and estimating the amounts of unreacted dipeptide with Cd-ninhydrin as recommended by Dryer & Bynum (11). The apparent pH profile of the condensa-

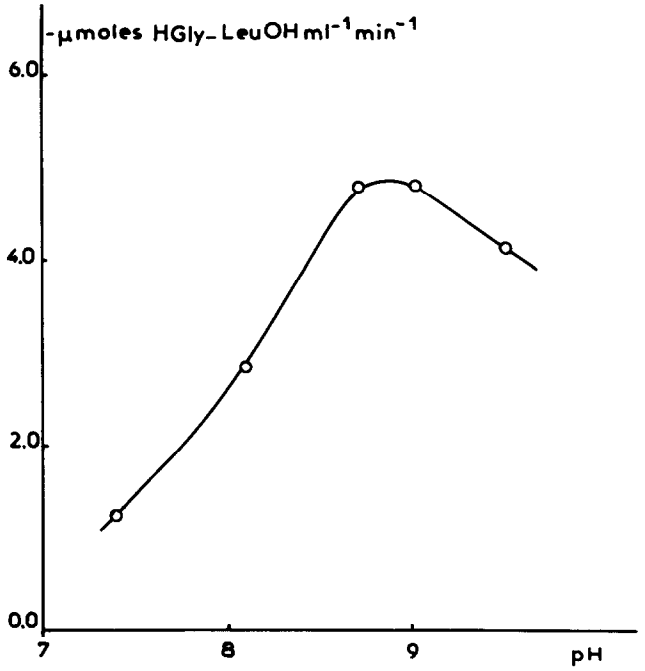

Fig. 1 - Condensation of CMDTB with HGly-Leu OH in function of pH. 6 μmoles dipeptide were incubated at 40°C with ca. 100 μmoles CMDTB in a total of 0.125 ml 40 % Pyridine in H_2O being 0.05 M in N-ethylmorpholine-acetate of various pHs. The corresponding initial velocities were estimated graphically from the progress curves of the reactions, obtained as described in the text.

tion could thus be delineated (Fig. 1) and also the time course at the optimum pH (Fig. 2). From these results, it is clear that CMDTB is the most interesting of the three reagents investigated, and that the reaction with H-Gly-Leu OH (Fluka) is complete in 1 h. at 40°C when examined under the above conditions.

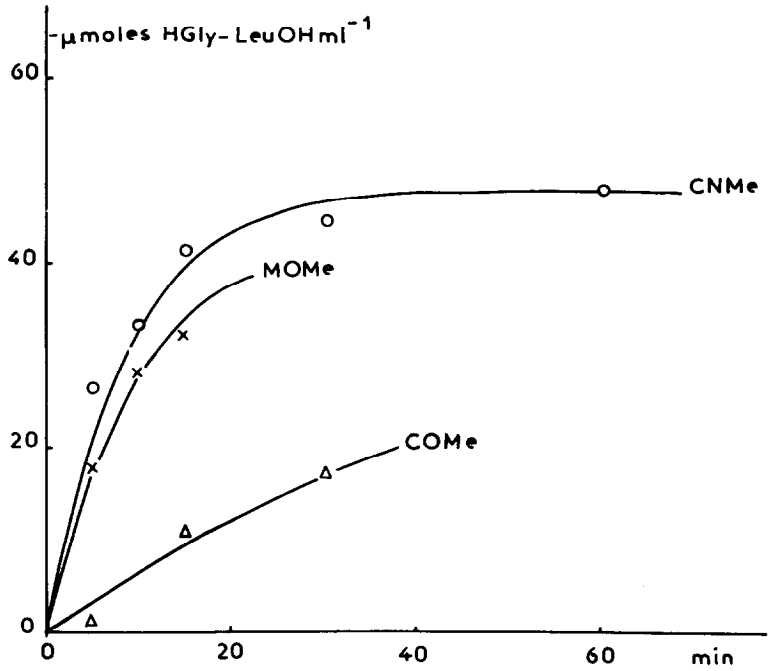

Fig. 2 - Condensation of various dithiobenzoates with HGly-LeuOH. 6 µmoles dipeptide were incubated at 40°C with ca. 100 µmoles ester in a total of 0.125 ml 60 % Pyridine in H_2O being 0.4 M in dimethylallylamine-trifluoroacetate pH 9.5 (5). The progress curves of the reactions were obtained as described in the text. CNMe = cyanomethyldithiobenzoate (97 µmoles), MOMe = methoxymethyldithiobenzoate (102 µmoles), COMe = carboxymethyldithiobenzoate (113 µmoles).

A comparison of the velocity of condensation of CMDTB or PITC with free amino groups was afforded by an investigation of their reaction with leucine, which permitted an estimation of the corresponding apparent kinetic constants at two temperatures (Table I). It is seen that CMDTB, although not so efficient a reagent for α-amino groups as PITC, nevertheless differs in reactivity from this last reagent only by a factor of about three, a difference which becomes insignifiant in practice when the coupling reagent is present in more than 50-fold excess over the amount of free amino groups.

Table 1

Apparent kinetic constants (ml μ moles^{-1} min^{-1}) for the rates of condensation of CMDTB and PITC with Leucine*

Temp.	CMDTB	PITC
23°C	0.22×10^{-3}	0.67×10^{-3}
43°C	1.06×10^{-3}	2.64×10^{-3}

* 6 μmoles amino acid were incubated at the two temperatures with known amounts (10-40 μ moles) of condensing agents in a total of 0.110 ml 60 % Pyridine in H_2O being 0.4 M in dimethylallylamine-trifluoroacetate pH 9.5 (5). From the progress curves of the reaction, obtained as described in the text, the apparent second order rate constants were calculated, neglecting the spontaneous hydrolysis of the condensing agents.

Furthermore CMDTB has a slightly more favourable temperature coefficient than PITC.

Cyclization step and sequential degradation of peptides.

When an aliquot from the various reaction mixtures of the dithiobenzoic esters with dipeptides was removed, taken to dryness in vacuo and treated with a minimum of anhydrous trifluoroacetic acid at room temperature, an amount of the second amino acid equivalent to the amount of condensed dipeptide was always found within 20 min. These results entirely confirm those described earlier by Barrett (7).

The sequential degradation of the pentapeptide H Phe-Asp-Ala-Ser-Val OH (Mann Research Laboratories) was thus attempted as follows. 1 µmole peptide in 100 λ of pyridine/water/N-ethylmorpholine (15 : 10 : 2.9) adjusted to pH 9.5 with acetic acid and 15 mg (78 µmoles) CMDTB were incubated at 51°C for 75 min. under nitrogen. Excess reagent was removed by five washings with 1 ml benzene, and the residue was lyophilized for 2 hrs. 20 λ trifluoroacetic acid were then added, and the solution was left for 30 min. at room temperature under nitrogen, when it was dried under a jet of nitrogen. The residue was extracted three times with 1 ml benzene after addition of 100 λ H_2O, dried in vacuo, and redissolved in 100 λ of the initial pyridi-

Table II

Sequential degradation of H Phe-Asp-Ala-Ser-Val OH by CMDTB *

	Phe	Asp	Ala	Ser	Val
Initial peptide	0.96	1.04	1.01	0.94	1.11
Rem. peptide after 1st st.	0.01	0.94	1.01	1.03	1.07
after 2d st.	0.01	0.19	1.16	1.12	1.05
after 3d st.	0.01	0.09	0.13	0.73	0.78
after 4th st.	0.01	0.09	0.09	0.25	0.39

* Results are expressed in μ moles amino acid and have been normalized to 1 μ mole at the initiation of each stage.

ne buffer above. 5 λ were removed for peptide analysis, and 5 λ for amino acid analysis after 22 h. hydrolysis in 6M HCl at 110°C in vacuo, both on the Beckman 120 B analyzer, and the remaining was recycled. The completeness of the condensation at each of the successive stages of the degradation could thus be ascertained by the complete absence, at its end, of any undegraded peptide, and the amino acid liberated could be identified by the substractive method which gave results (Table II) in excellent agreement with expectation.

Conclusion

CMDTB thus appears to hold promise of being a reagent suitable for the sequential degradation of peptides from their amino end, in a manner similar to that afforded by the Edman procedure (4). However, it presents some distinct advantages with respect to this last technique, because the 2-phenylthiazolinones formed after the cyclization step are more easily generated, are stable, and represent the terminal product of the reaction, in contradistinction to the 2-anilino-thiazolinones of the Edman reaction. This, in the first place, leads to the possibility of performing the cyclization step under milder conditions. Secondly, the three steps of the degradation (12) are reduced to two steps, as no isomerisation is required. These points have also being recognized by Barrett (7).

It is possible that the conditions described in the present communication could be improved. Further, the condensation of free amino groups with nitrophenyl dithiobenzoates, which can likewise be prepared in crystalline form, could be more rapid, but problems relating to the solubility of the reagents and to the possibility of undesirable side reactions have to be examined. Also, for application in automatic instruments (2, 3), where the cyclization step ought to be carried out at the same higher temperature as the coupling step, a less strong acid could probably be substituted to trifluoroacetic or heptafluorobutyric acid, so that the risk of spurious internal cleavages is still avoided. Finally CMDTB might be covalently included into a polystyrene matrix in a way similar to that developped for peptide synthesis (9) or for the Edman degradation itself (13).

It can be expected, in addition, that the acid hydrolysis of the phenylthiazolinones shall yield the parent amino acid in better yields than can be obtained from the phenylthiohydantoins. Preliminary experiments in this direction (6M HCl, 110°C) have shown that this is indeed the case,so that the use of the automatic amino acid analysis techniques available today appears to be open for the rapid and unequivocal identification of the released N-terminal amino acid derivatives, a fact which would obviate the necessity for laborious manipulations and chromatographies. It should also be noted that the phenylthiazolinones of amino acids are highly fluorescent in aqueous medium above pH 7 when viewed under UV-light.

Further investigations in these different directions are presently being carried on.

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