BASE CATALYZED AMINOLYSIS OF CARBODITHIOIC ESTERS AND ITS INTEREST IN SOLID PHASE SEQUENTIAL ANALYSIS OF PEPTIDES

A. PREVIERO, A. GOURDOL, J. DERANCOURT and M.-A. COLETTI-PREVIERO

Groupe de Recherches U-67 (INSERM), Centre de Recherches de Biochimie Macromoléculaire (CNRS), BP 5051, 34033-Montpellier, France

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1. Introduction

The finding that a peptide terminal *N*-thioacyl derivative can be split off by the action of TFA yielding the 2-substituted thiazol-5(4H)-one which identifies the N-terminal amino acid and the shortened peptide constitutes the base of a new route for the sequential analysis of polypeptide chains [1–3]. The mild reaction conditions required for the release of the N-terminal thiazolinones together with the chemical and physical properties favourable for their identification constitute the most promising aspect in view of a complete automatization of the analytical procedure.

The thioacylation reaction is brought about by carbodithioic esters (sometimes by thionic esters) because there are no other generally satisfactory reagents for thioacylating amino groups. Nevertheless these compounds are not particularly good thioacylating reagents and this constituted the most important drawback for a satisfactory procedure of sequential analysis.

In order to overcome this difficulty the use of active esters of dithiobenzoic acid has been proposed [2] and the results obtained led one to recognise that some esters containing a good leaving group could be usefully choosen after a systematic exploration.

An alternative possibility of general application to increase the rate of the coupling reaction between amino groups and thioacylating reagents is proposed in this work. We report here that aminolysis of carbodithioic esters is susceptible to base catalysis and we describe the experimental conditions by which simple methyl esters of aliphatic dithioacids behave as strong thioacylating reagents. These conditions are compatible with the ones required for an automatic sequential analysis of peptides especially by the solid-phase technique [4].

2. Experimental

Dithioesters were obtained by thiohydrolysis of *S*-methylthiomorpholinium halides [5] or *S*-methylthiopiperidinium halides [6]. I. Methyldithioacetate Thioacetylpiperidine [6] (0.5 mol) and methyliodide (42 ml) were dissolved in dry benzene (250 ml) and the solution allowed to stand about 24 hr at room temperature. *S*-methyl-thioacetylpiperidinium iodide, which separates as a crystalline product, was collected by filtration, washed with ethylether, and utilized without further purification. This last product was dissolved in methanol (200–250 ml) and the solution maintained saturated with H_2S for about 24 hr at room temperature. The solution was then diluted with an excess of water (about 1500 ml) and extracted with ethylether. The organic layer was dried (Na_2SO_4) and the ether removed under vacuum heating not higher than 20–25°C. The remaining yellow liquid was distilled under reduced pressure and methyldithioacetate collected at 40–41°C/15 mm in 28% yield.

$\lambda_{max}^{ethanol}$ (log ϵ) = 302 (4.11)

Anal. calcd. for $C_3H_6S_2$: C, 33.93; H, 5.69; S, 60.38
found: C, 33.98; H, 5.72; S, 60.15

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II. Methyldithiopropionate (b.p. 55-56°C/15 mm), III. Methyldithiobutyrate (b.p. 70-72°C/15 mm) and IV. Methyldithioisobutyrate (b.p. 62-63°C/15 mm), were similarly obtained starting from the corresponding thioacylpiperidine derivatives.

2.1. *Kinetic measurements*

Stock solutions of carbodithioic acid esters and amino compounds were freshly prepared for each series of runs in appropriate solvents. The other materials utilized in the different experiments were also prepared in stock solutions. The reaction mixtures were made up by mixing requisite quantities of the thermostated stock solutions and solvent to obtain the desired starting concentrations.

The extent of thioacylation reaction was followed on one hand by measuring at 263 nm (λ max thioamide, log ϵ = 4.10) suitable aliquots diluted in acidic ethanol and on the other by the decrease of free amino groups using ninhydrin method. The pseudo first order reaction constants were calculated from the formula $k = \frac{2.303}{t} \log \frac{a}{a-x}$. The initial concentration of the reactants was the same in all cases.

2.2. *Stepwise degradation of peptides*

2.2.1. Insolubilisation of peptides.

N-Boc peptides (about 200 nmoles) were attached to methyl triethylene tetraminopolystyrene (100 mg) through their terminal carboxyl group by the general procedure previously described [4]. The resulting resin was introduced into the column of an automatic sequencer, similar to the one described by Laursen [7] and constructed by one of us (J.D.), and then treated with TFA to remove the *N*-blocking group.

2.2.2. Condensation step

A solution of methyldithioacetate: triethylammonium acetate 1 M in DMF (1:9) was passed (5 ml/hr) through the insolubilised peptide for 40 min at 45°C. The resin was then alternatively washed twice 4 min with methyl alcohol and dichloroethane (50 ml/hr) both containing 0.2% of mercaptoethanol.

2.2.3. Cyclisation step

TFA (3.4 ml/hr) was passed through resin for 25 min and collected. The TFA was evaporated under nitrogen stream and the residue was submitted to automatic amino acid analysis after acid hydrolysis (2 N HCl, 5 hr, 110°C). The residual resin was alternatively washed with dichloroethane (4 min) and methyl alcohol (10 min) and then submitted to the subsequent cycle of degradation. The first fraction (about 1 ml) of dichloroethane washing which still contains some thiazolinone was added to TFA fraction before evaporation.

3. Results and discussion

A series of runs was made in which leucine methylester (LeuOMe) was allowed to react with 20-fold excess of carbodithioic esters in different solvents to which basic catalysts were added. The large excess of thioacylating reagent has been choosen since the aim of the present work was to find the experimental conditions for a rapid thioacylation of amino terminal groups of polypeptides in order to bring about sequential degradation.

The thioacylation of LeuOMe with methyldithioacetate takes place very slowly indeed (fig.1, curve a). The effect of adding some basic catalysts such as a tertiary amine or an acetate anion is to increase the

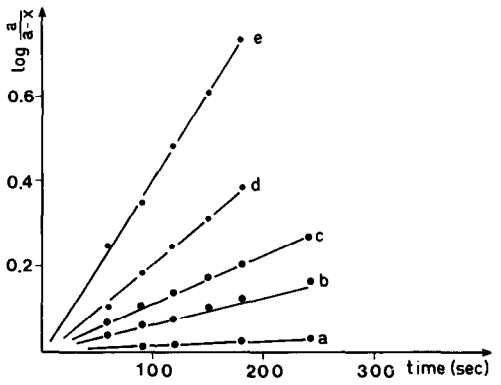
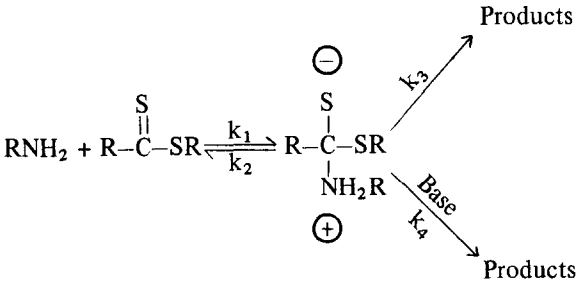


Fig.1. Thioacetylation of LeuOMe in DMF with various compounds as catalysts.
[LeuOMe] = 0.05 M; [CH_3CSSCH_3] = 1 M; temperature = 20°C.
a) without catalyst. b) triethylamine 0.5 M. c) triethylammonium acetate 0.1 M. d) triethylammonium acetate 0.2 M. e) triethylammonium acetate 0.5 M.

values of the rate of the reaction. The straight lines plotted in fig.1 show quite clearly that the thioacetylation reaction, using at least 20-fold excess of carbodithioic ester, may be represented as a pseudo monomolecular reaction. Triethylamine and triethylammonium acetate behave as catalyst in that they speed up the reaction, but do not change its order.

These results suggest that the aminolysis of carbodithioic esters is similar to the one of carboxyl esters, which also exhibits general base catalysis [8]. In other words, the reaction should proceed simultaneously by two pathways, one catalyzed by a base which can be a tertiary amine or a carboxylate anion and the other not.



The results reported in table 1 show that the anion of a weak carboxylic acid (i.e. acetic acid) is a more efficient catalyst than anions of stronger acids or tertiary amines.

The solvent is another important factor in determining the rate of aminolysis of dithioacid esters. The kinetic changes of the *N*-thioacetylating reaction in different non aqueous solvents are reported in table 2. A minimum amount of catalyst (triethylamine 0.2 M) was utilized in order to evidence the solvent effect.

Table 1

Catalyst 0.5 M	K × 10^4 sec^{-1}	
	Photometric run*	Chemical run**
$CH_3COOHN(C_2H_5)_3$	91.2	90.8
$HCOOHN(C_2H_5)_3$	86.4	85.4
$CF_3COOHN(C_2H_5)_3$	13.8	11.9
$N(C_2H_5)_3$	15.8	16.5

Effect of different carboxylate anions and triethylamine on the reaction rate between methyldithioacetate and LeuOMe in DMF.
[CH_3CSSCH_3] = 1 M; [LeuOMe] = 0.05 M; temperature = 20°C.

* Increase of adsorbance at 263 nm (thioamide formation)
** Ninhydrin method (disappearance of LeuOMe)

Table 2

Solvent	$t_{0.5}$ sec
Methanol	131
Dimethylformamide	444
n-Butanol	517
Hexamethylphosphoric triamide	1216
Dioxane	6930

Thioacetylation of LeuOMe in different solvents.
[CH_3CSSCH_3] = 1 M; [LeuOMe] = 0.05 M;
catalyst: triethylamine 0.2 M; temperature = 20°C.

The rates of reaction of carbodithioic esters with primary amines are also dependent upon the structure of the carbodithioic esters, being affected by inductive and steric effects. The reactivity of homologous dithioesters shows the following order: $CH_3CSSCH_3 > CH_3CH_2CSSCH_3 > CH_3CH_2CH_2CSSCH_3 > (CH_3)_2CHCSSCH_3$ (fig.2).

As for the use of aliphatic carbodithioic esters in sequential analysis of polypeptide chains, two major considerations must be made. The first one is that the

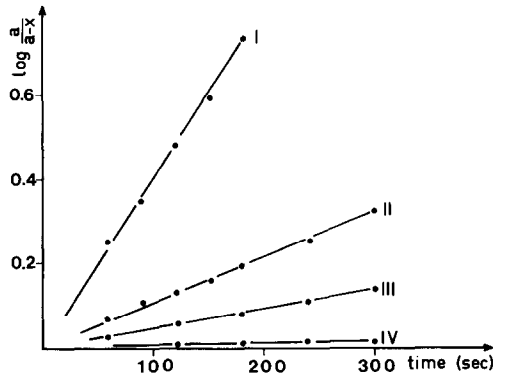
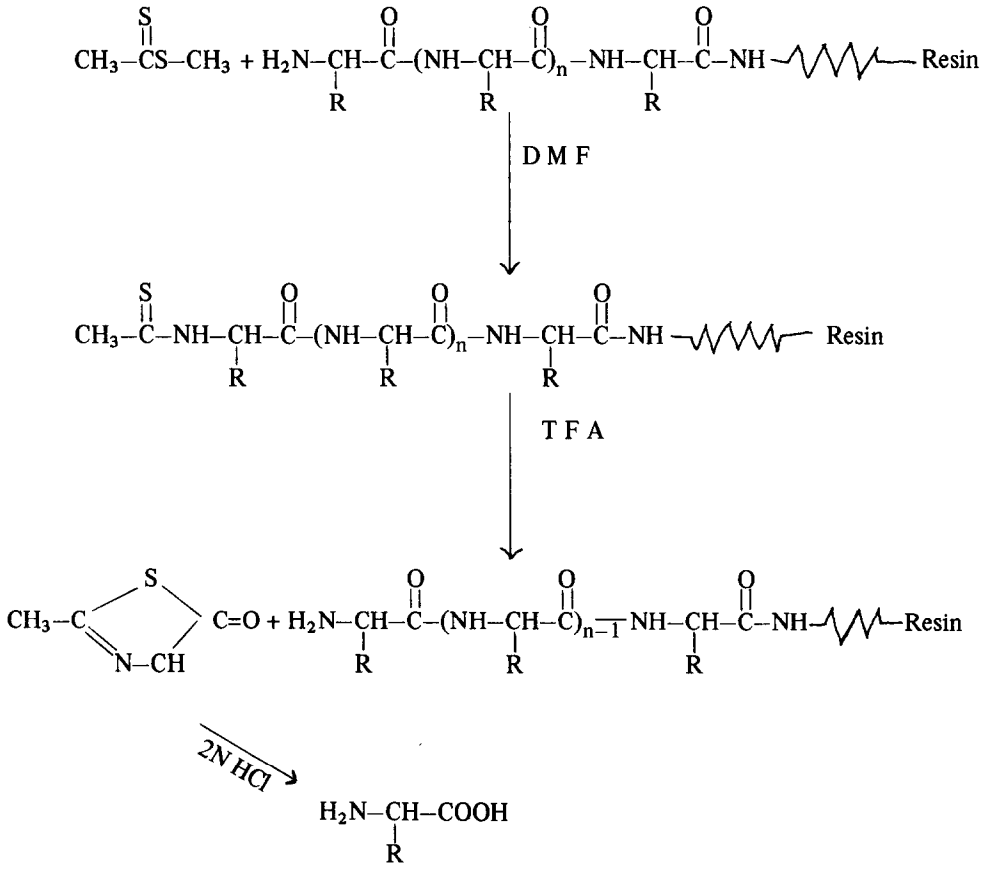


Fig.2. Reaction of LeuOMe with different carbodithioic esters ($RCSSCH_3$) in dimethylformamide.
[R $CSSCH_3$] = 1 M; [LeuOMe] = 0.05 M.
Catalyst: triethylammonium acetate 0.5 M; temperature = 20°C.
R = CH_3 (I); R = CH_3CH_2 (II); R = $CH_3CH_2CH_2$ (III); R = $(CH_3)_2CH$ (IV).

most efficient catalysts which must be used to bring about the thioacylation of the N-terminal group of peptides are trialkylammonium salts, poorly or not volatile, so that an undesired accumulation of non-peptidic material with the increase of degradation steps is practically inevitable unless some machinous manipulations are introduced. The second one concerns the slight solubility of carbodithioic methyl or ethyl esters in aqueous solvents (methyldithioacetate exhibits a solubility of about 5% in ethanol:water, 1:1). Furthermore, the catalysts for aminolysis of the dithioesters are probably efficient to increase also their rate of hydrolysis which could become a too important competitive reaction.

The sequential degradation by solid-phase technique [7] seems to overcome all these difficulties. Indeed only washing by filtration is utilised instead of evaporation and the sequential degradation can be performed entirely in non-aqueous media [4]. The choice of the solvent for the condensation step with the N-terminal of a peptide insolubilised by linking to a solid support is dependent from the nature of the support concerned. In the case of polystyrene resins, the dimethylformamide is the better solvent for a good swelling of the resin as well as for a satisfactory rate of thioacylating reaction (table 2).

Some peptides, linked to a polystyrene matrix by their C-terminal group [4] were submitted to sequential degradation in heterogeneous phase by a procedure summarized in the following scheme:



As suggested by kinetic experiments in homogeneous phase, methyl dithioacetate (fig.2) was utilised as reagent and triethylammonium acetate (fig.1) as catalyst in order to increase the rate of the thioacylating reaction. Time and temperature were largely sufficient for a complete thioacylation. Reductive reaction conditions are strongly recommended to avoid the desulfurisation of the formed thioamides to the corresponding improductive amide [9]. These reductive conditions are partially secured by the liberation of the methylmercaptan which parallels the condensation reactions, but thioalcohols (i.e. mercaptoethanol) should be added especially to the washing solvents. The subsequent cyclisation step which results in the release of the N-terminal residue as methylthiazolinone, easily takes place by TFA treatment in heterogeneous phase as already observed on insolubilized peptides thioacylated with carboxymethyldithio acetate [10]. Stepwise degradation was performed on the peptides: Gly–Leu–Phe, Phe–Asp–Ala–Ser–Val, Phe–Val–Glu–Trp–Leu–Met–Asn–Thr with approx. 90% yield per cycle.

The identification of released thiazolinones was performed through the regeneration of the amino acid. This identification fails in the case of Ser, Thr and Trp as well as in distinguishing between Asp–Asn and Glu–Gln, but other analytical procedures (i.e. gas-liquid chromatography or thin-layer chromatography of methylthiazolinones) should overcome such limitation.

It is our opinion that the different kinds of insoluble supports [7,11,12] utilisable in the solid phase technique together with the possibility to bring about sequential degradation of peptides entirely within purely organic solvents should permit to multiply the chemical possibilities for the choice of the reagents as well as for the analytical identification of the amino acid derivatives to reach a complete automatized procedure.

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