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DERIVATIZATION OF AMINO ACIDS WITH 1,3-DICHLOROTETRAFLUOROACETONE AND ITS USE IN GAS CHROMATOGRAPHY

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SUMMARY

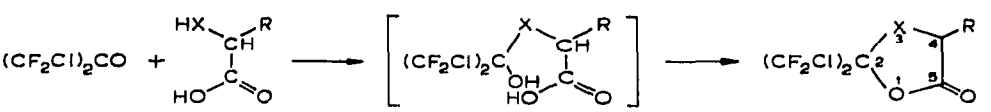
In a weakly basic non-aqueous medium a condensation reaction proceeds between dichlorotetrafluoroacetone and an α-amino acid, such that two adjacent protonic groups, amino and carboxylic, were coupled together with the ketone in a relatively stable five-membered ring. The resulting cyclic derivative, 2,2-bis(chlorodifluoromethyl)-4-subst.-1,3-oxazolidin-5-one was of essentially lower polarity and could be used for gas chromatography. With regard to a possible application in the analysis of both protein and thyroid amino acids, the reaction conditions were tested on tyrosine and its iodinated homologues, mono- and diiodotyrosines. The hydroxyl group of the benzene ring was esterified by treatment with heptafluorobutyric anhydride in the second reaction step.

INTRODUCTION

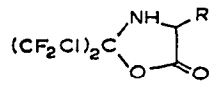
The use of halogenated acetones offers new possibilities in the estimation of α-amino acids by gas chromatography. The presence of the highly electro-negative fluorine and/or chlorine atoms enhances the acidic character of the carbonyl group so that, in contrast to the behaviour of aliphatic ketones, stable adducts with water, aliphatic alcohols, and ammonia are formed. In the case of 1,3-dichloro-1,1,3,3-tetrafluoroacetone (DCTFA), the equilibrium of the reaction

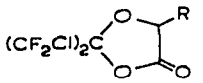
$$(CF_2Cl)_2C{=}O + H{-}X \rightleftharpoons (CF_2Cl)_2\overset{OH}{\overset{|}{C}}{-}X \qquad (X = -OH, -OR, -NH_2)$$

is markedly shifted to the right. With α-substituted carboxylic acids, a stable cyclic derivative is formed, in which the two adjacent polar groups, the α-protonic (amino, hydroxy or thiol) and the carboxylic, are coupled in a five-membered ring by a condensation reaction:



Substitution of X with –NH, –O, or –S resulted in 2,2-bis(chlorodifluoromethyl)-4-subst.-1,3-oxazolidin-5-one (I), -dioxolan-5-one (II), or -oxathiolan-5-one (III), respectively.


I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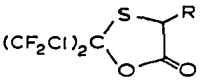
III

Considering the estimation of such derivatives by the electron capture detector (ECD), the use of DCTFA seems to be very promising. There was a report[1] about its photochemistry in 1972. The two chlorine and four fluorine atoms should contribute considerably to ECD response. Moreover, the boiling point of DCTFA (45°) allows easy evaporation after the reaction is completed. The other halogenated acetone which could be considered, the hexafluoroacetone, is a gas, is more expensive, and does not contribute to the ECD response to the same extent as DCTFA.

The reactions of the halogenoacetones were studied extensively by Simmons and Wiley[2]. In their report, preparation of the oxazolidinone from alanine is described. In addition, Engelhardt[3] and Weygand and his colleagues[4–6] have successfully prepared the oxazolidinones from some simple protein amino acids and chromatographed them. However, there were difficulties in dissolving the acids in the reaction medium, as the acids themselves are not soluble in DCTFA. In a solvent like acetonitrile, the conversion of amino acids to oxazolidinones occurred at elevated temperature after several hours. Moreover, in the case of amino acid hydrochlorides, no conversion was observed under the same reaction conditions. With respect to the fact that only hydrochlorides are formed after hydrochloric acid hydrolysis of proteins, the use of this method was limited.

The aims of this report were to find the optimal reaction conditions for the quantitative conversion of amino acids (including their hydrochlorides) to oxazolidinones and to examine the chromatographic properties of these derivatives. As representatives of protein and thyroid amino acids, tyrosine and its mono- and diiodinated homologues 3-iodo-L-tyrosine and 3,5-diiodo-L-tyrosine were chosen.

EXPERIMENTAL

Reagents

DCTFA (d_4^{20} = 1.52, b.p. 45.2°; pure), 3,5-diiodo-L-tyrosine (DIT) (pure), and N-methylpyrrolidine (pract.) were purchased from Koch-Light (Colnbrook, Bucks., Great Britain); acetonitrile, pyridine, α-picoline, β-picoline, triethylamine, N-methylpiperidine, dimethylformamide (DMFA), dimethyl sulphoxide (DMSO), heptafluorobutyric anhydride (HFBA), and L-tyrosine (all chemicals of analytical grade) from Merck (Darmstadt, G.F.R.); 3-iodo-L-tyrosine (MIT) from Sigma (St. Louis, Mo., U.S.A.); and lindane (GLC grade) from Supelco (Bellefonte, Pa., U.S.A.). The chemicals were stored in a refrigerator at 4°. DCTFA could be used for some months without treatment; after this period phosphorus pentoxide treatment and distillation are recommended.

Apparatus

The reactions were carried out in stoppered 3-ml glass tubes or in 1-ml reaction vessels with Teflon-lined caps (Supelco). The oxazolidinones of tyrosine, MIT and DIT were analysed together with lindane as internal standard by a Chrom-2 gas chromatograph (Laboratory Equipment, Prague, Czechoslovakia) that was equipped with a dual flame ionization detection system and fitted with a linear temperature programmer. The glass column, 1.8 m × 3 mm I.D., was filled with 5% SE-30 (Varian-Aerograph, Palo Alto, Calif., U.S.A.) on Chromaton N-AW-HMDS (Lachema, Brno, Czechoslovakia). The temperature was programmed from 180 to 220° at 2.5°/min. Detector and inlet temperatures were set at 240°; the nitrogen carrier gas flow-rate was 20 ml/min, and the chart paper rate was 30 cm/h.

Procedure

For examination of the reaction conditions, the amino acids were used in three different forms: (1) as free acids (weighed as solids); (2) as ammonium salts dissolved in methanol–10% ammonia (9:1); (3) as hydrochlorides dissolved in 96% ethanol–1 *N* aqueous HCl (2:1). For the experiments, all these forms were used. The stock solution (10 ml) contained 0.2 mmole tyrosine, 0.2 mmole MIT, and 0.6 mmole DIT. In each experiment, 100 to 250 μl of the solution were evaporated at 50° under a stream of nitrogen and dichloromethane was then added to remove traces of water azeotropically.

In order to find optimal conditions for the condensation reaction, the influence of solvent, catalyser, time and temperature, dilution, and excess of reagent were studied; for details of each procedure, see the text under each figure. After the first reaction step the oxazolidinones were treated with HFBA (20 μl of HFBA were added to the aliquot of the first reaction and the sample was kept at 50° for 15 min) to esterify the phenolic group. Before injection into the column, an aliquot of lindane in toluene was added, so that the amount injected was 1.25 nmole of lindane, 2 nmoles each of tyrosine and MIT, and 6 nmoles of DIT in a volume of 2 μl. They were eluted from the chromatographic column in the above order.

RESULTS AND DISCUSSION

At first, a convenient solvent had to be found, as the amino acids themselves were not soluble in DCTFA. Earlier attempts[2,3] were made with DMFA or DMSO (13.4 g alanine treated with 60 g DCTFA and 50 ml DMFA at 60° for 3 h gave oxazolidinone yields of about 83%). The use of DMSO had one advantage: if oxazolidinones were extracted with an organic solvent (*e.g.*, dichloromethane), DMSO remained fully in the water phase and so could be easily removed. Later, the use of acetonitrile was reported. The respective yields of oxazolidinones from some protein amino acids at elevated temperatures (70–90°) and prolonged reaction time were greater than 90%.

Various organic solvents were evaluated in our search for a suitable one, although only DMFA, DMSO, pyridine, and acetonitrile appeared to be of practical use. In the presence of DCTFA the amino acids were best dissolved in DMSO even at room temperature; in DMFA and pyridine the acids were dissolved within 5 min at 50°. In acetonitrile only a part was dissolved at 50°. Chromatographic analysis

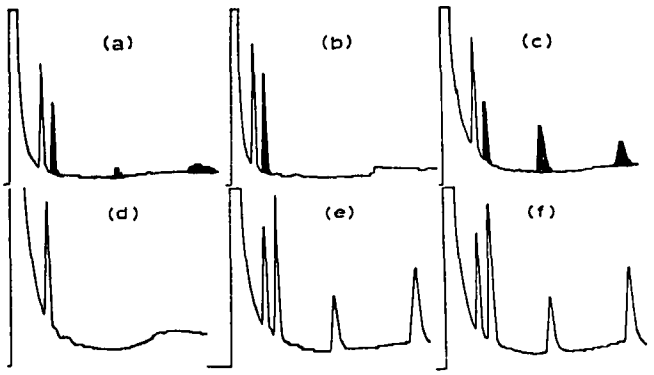

Fig. 1. Influence of various solvents (250 μl added) on the reaction (15 min at 50°) between 50 μl of DCTFA and 25 μmoles of amino acids. The black peaks represent non-quantitative conversion. (a) Dimethylformamide; (b) dimethyl sulphoxide; (c) acetonitrile; (d) pyridine; (e) acetonitrile–pyridine (20:1); (f) acetonitrile–pyridine (100:1).

(Fig. 1) demonstrated clearly that in acetonitrile this dissolved portion was simultaneously converted to oxazolidinones. In the other solvents used hardly any oxazolidinones were found (the black peaks in the figures represent only partial conversion). Since only acetonitrile appeared to support oxazolidinone formation, our interest was turned to it. Various organic bases were tried in order to improve the dissolving ability of acetonitrile. The addition of a small amount of a weak organic base (pyridine and/or its methylated forms, the picolines) into the acetonitrile improved the solubility remarkably. A ratio of base to acetonitrile of 1:100 was satisfactory to achieve rapid

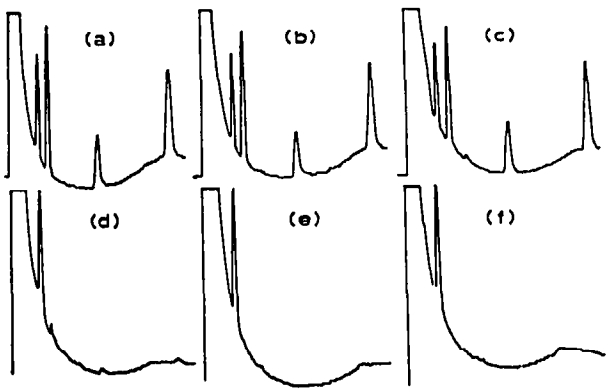

Fig. 2. Influence of different bases diluted 1:100 with acetonitrile (250 μl) on the reaction between 50 μl of DCTFA and 25 μmoles of amino acids (15 min at 50°). (a) Pyridine; (b) α-picoline; (c) β-picoline; (d) N-methylpiperidine; (e) N-methylpyrrolidine; (f) triethylamine.

solution and conversion of all the acids, irrespective of whether the free acids or their hydrochlorides were applied. It was surprising that after addition of strong nitrogen bases, *i.e.* tertiary amines, no oxazolidinones were formed (Fig. 2). Moreover, a decomposition of DCTFA by those strong bases was observed.

The effect of time and temperature on the condensation reaction is apparent

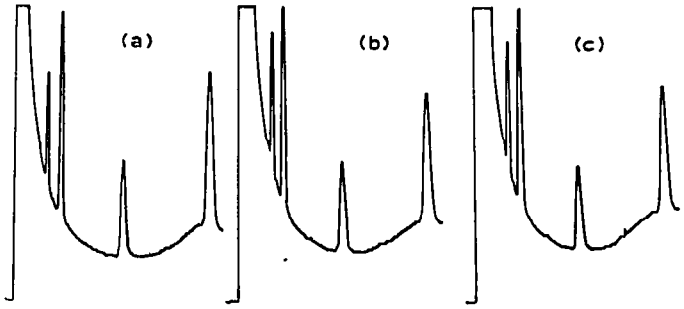

Fig. 3. Influence of temperature and time on the reaction. In each experiment 10 μmoles of amino acids were treated with 20 μl of DCTFA, and 100 μl of the solvent (acetonitrile–pyridine, 100:1). The first sample (a) was analysed immediately after dissolving the acids at room temperature (at 20° for 5 min), the others (b and c) were heated at 20° for 5 min plus 50° for 10 min and at 20° for 5 min plus 100° for 20 min, respectively.

from Fig. 3. The simple shaking of the sample at 20° till the acids were dissolved led to the complete conversion into oxazolidinones. No other changes in yield were observed after heating. This fact confirms the findings demonstrated in Fig. 1 that in acetonitrile as a reaction medium, the dissolved fraction of amino acid is simultaneously converted.

Also the ratio of DCTFA to solvent (Fig. 4) as well as of DCTFA to the amount of acids (the molar ratio, Fig. 5) were altered. Dilution of 1:20 had no effect on the results; a decrease to 1/3 of the original 15-fold molar excess led to a decrease in yield but its further diminishing to 1/5 caused the loss of the yield. However, in this last case the added molar amount of pyridine was smaller than that of amino acids (or their hydrochlorides), so that dissolving of the acids did not occur.

The same results were obtained with amino acids and their hydrochlorides. The trace amount of pyridine was added, therefore, to acetonitrile not only to remove the hydrochloric acid but because the presence of the organic base generally improved the dissolving ability of acetonitrile for the amino acids. In this way the reaction time could be shortened from several hours to a few minutes.

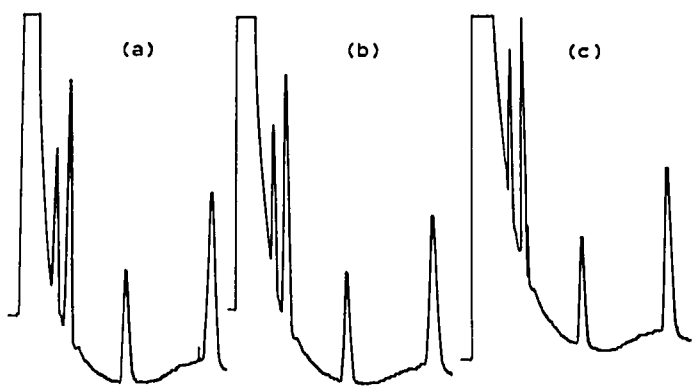

Fig. 4. The DCTFA dilution (in the earlier experiments 1:5) was varied from (a) 1:10 to (b) 1:15 to (c) 1:20, *i.e.* to 13 μl of DCTFA, 130, 200 or 260 μl of the solvent were added. The reaction with 10 μmoles of amino acids was carried out at 20° for 5 min.

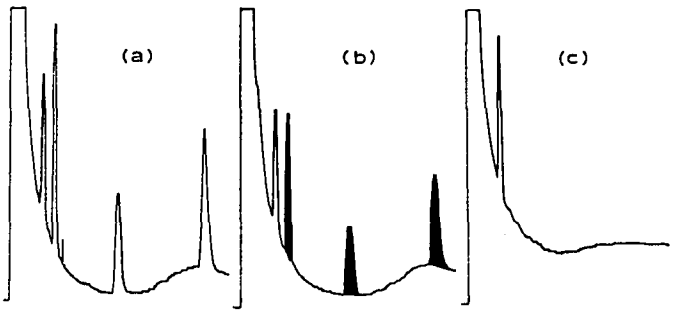

Fig. 5. The DCTFA/amino acid molar ratio (in the earlier experiments 15) was diminished to (a) 7.5:1, (b) 5:1, (c) 3:1. The DCTFA dilution was kept constant at 1:15. Thus, 150, 100 and 60 μl of the solvent, respectively, and 10, 6.5 and 4 μl of DCTFA, respectively, were added to three reaction vials, each containing 10 μmoles of amino acid, and the mixture was shaken for 5 min at 20°.

The condensation reaction with DCTFA led to the closing of the ring between the α-amino and the carboxylic group. Formation of a Schiff base as a possible by-product was found to be very unlikely, due to the strong competition of the carboxylic proton in the neighbourhood. Moreover, evidence was found that removing the second proton of the amino group is rather difficult. Under the same reaction conditions, the methyl esters of α-, β-alanine and phenylalanine were treated with DCTFA. Even after 30-min heating at 50°, no conversion of the amino group to the Schiff base was observed.

There was another point to be explained. For the amino acids treated in this paper, a second reaction step was required: the esterification of the remaining hydroxyl group to enable their analysis by gas–liquid chromatography. The added anhydride (in this case HFBA) is a powerful dehydration agent, which could induce closing of the ring in the condensation reaction. Is, therefore, addition of the anhydride necessary for this purpose or is the treatment with DCTFA sufficient for the complete closing of the oxazolidinone ring?

An attempt was made to answer this question. The ^{131}I-labelled iodothyronines T_3 and T_4 (Radiochemical Centre, Amersham, Great Britain) were developed after the first reaction (5 min at 50°) by chloroform on a thin layer of silica gel[7]. The radioactive peaks were then scanned by the Berthold scanner II. More than 95% of the radioactivity was associated with the leading oxazolidinone spot. In most cases complete conversion to the oxazolidinones was found. If lower yields were obtained, *e.g.* after longer storage of DCTFA, the addition of anhydride (trifluoroacetic anhydride or HFBA) is recommended. In any case (as in the case of trifluoroacetylated esters) strong anhydrous conditions are required for the oxazolidinone formation.

In spite of the fact that the amino acids were converted to the oxazolidinones in relatively short times, the rate of conversion was a little different. The two iodinated homologues were dissolved (and therefore converted) in the reaction medium more easily and more rapidly than tyrosine. Preliminary experiments carried out with the iodinated thyronines and with the protein amino acids showed clearly that the former dissolved in the medium easily even at room temperature, whereas the latter dissolved with difficulty. However, the application of the method to these two biochemically very important groups will be the subject of another paper.

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