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GAS CHROMATOGRAPHIC BEHAVIOUR OF AMINO ACID OXAZOLIDINONES

RESPONSE TO FLAME IONIZATION AND ELECTRON CAPTURE DETECTORS

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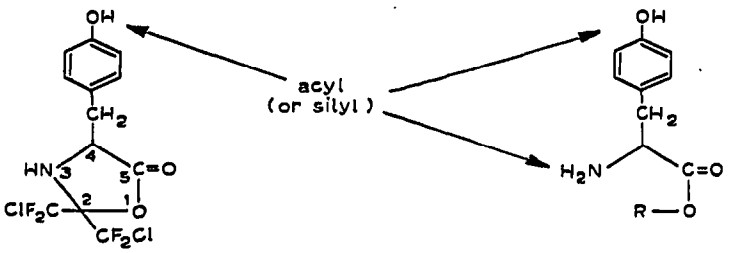
SUMMARY

The use of 1,3-dichloro-1,1,3,3-tetrafluoroacetone for derivatization of α-amino acids offers two important advantages. Firstly, the amino group, which is coupled with the carboxylic group in a five membered ring, does not require further treatment with an anhydride or silyl reagent and, secondly, the next reaction step, the esterification of the remaining protonic groups, can be performed immediately after the first reaction without evaporation of the reaction medium. Moreover, the oxazolidinones show very good chromatographic properties and a high response to the electron capture detector (ECD) comparable with that of lindane. The response to the flame ionization detector (FID) is nearly the same as that of trifluoroacetylated methyl esters of amino acids. The oxazolidinones of tyrosine and mono- and diiodotyrosines were prepared and, after treatment with acetic, trifluoroacetic, heptafluorobutyric or pivalic anhydride or with hexamethyldisilazane, they were chromatographed on silicone phases. The relative molar responses to hexadecane (FID) and lindane (ECD) were determined.

INTRODUCTION

Two steps are usually required for the preparation of chromatographically convenient derivatives of amino acids: (a) esterification of the carboxylic group with an aliphatic alcohol, followed by evaporation of the reaction medium, and (b) acylation (or silylation) of the other proton-carrying group (–NH_2, –OH, –SH) in order to decrease the polarity and increase the volatility of the derivative.

Another approach to the derivatization of amino acids is the use of halogenated acetones. A condensation reaction between the ketone and the amino and carboxylic groups leads to formation of a stable five-membered ring, the oxazolidinone. Thus, the amino group is bound rigidly in the cyclic ring and does not require further treatment as it does in the case of amino acid alkyl esters. Considering tyrosine and its iodinated homologues 3-iodo-L-tyrosine (MIT) and 3,5-diiodo-L-tyrosine (DIT), which after treatment with 1,3-dichloro-1,1,3,3-tetrafluoroacetone (DCTFA) yield the 2-bis(chlorodifluoromethyl)-oxazolidin-5-ones, the advantage of oxazolidinone



I II

(I) O-(Mono)acyl(silyl) oxazolidinone of tyrosine
(II) N,O-Diacyl(silyl) alkyl ester of tyrosine

formation in comparison with the carboxy esters is obvious. Before chromatography, the oxazolidinone (I) of an amino acid requires further treatment only if another protonic group is present, whereas its carboxy ester (II) always requires treatment with an anhydride (the example is shown for tyrosine). Moreover, for the latter evaporation of the esterification medium is necessary, whereas the former can be treated without evaporation.

The first detailed report dealing with the behaviour of halogenoacetones with regard to the reaction with α-substituted carboxylic acid was presented by Simmons and Wiley[1]. Later, Engelhardt[2] and Weygand and his colleagues[3,4] described the use of DCTFA for the preparation of 2-bis(chlorodifluoromethyl)-oxazolidin-5-ones of some protein amino acids. After treatment with hexamethyldisilazane (HMDS) some of these derivatives were analysed by gas chromatography. However, the derivatization conditions were rather strong (*i.e.*, several hours at elevated temperature) and the amino acid hydrochlorides could not be converted into oxazolidinones. Recently, there was a report[5] on the reaction of certain simple peptides (glycine peptides and glycine esters) and related compounds with hexafluoroacetone in dimethyl sulphoxide to afford fluorinated products containing an oxazolidinone ring. Further, the formation of 2-trifluoromethyl-oxazolin-5-ones as inner esters of N-trifluoroacetyl amino acids has been reported[6,7]. Several protein amino acids were treated with trifluoroacetic anhydride at 150° for 10 min and the resulting derivatives were said to have excellent chromatographic properties.

In the preceding study[8], the optimal reaction conditions were described for the rapid and easy preparation of the 2-bis(chlorodifluoromethyl)-oxazolidin-5-ones of tyrosine, MIT and DIT, which were chromatographed after treatment with heptafluorobutyric anhydride (HFBA). As well as HFBA, some other anhydrides, *e.g.* acetic (AA), trifluoroacetic (TFAA) and trimethylacetic (TMAA), and HMDS were used in this study for the esterification of the phenolic group. The gas–liquid chromatographic (GLC) properties of these compounds were tested by flame ionization (FID) and electron capture (ECD) detectors using hexadecane and lindane as internal standards. The relative molar responses were evaluated and compared with those of diacylated methyl esters.

EXPERIMENTAL

Reagents

DCTFA was purchased from Koch-Light (Colnbrook, Bucks., Great Britain)

or Fluka (Buchs, Switzerland). It was used in the commercial form for some months, but subsequently distilled with phosphorus pentoxide at 45 °.

The amino acids tyrosine (p.a.), MIT (p.a.) and DIT (pure) were supplied by Merck (Darmstadt, G.F.R.), Koch-Light, and Sigma (St. Louis, Mo., U.S.A.), respectively. Lindane (GLC grade) was purchased from Supelco (Bellefonte, Pa., U.S.A.) and hexadecane was a gift from the Institute of Chemical Technology in Prague.

Some of the anhydrides, *i.e.* TFAA and HFBA, and acetonitrile, pyridine, and other organic solvents were supplied by Merck. AA and the silylating reagents HMDS and trimethylchlorosilane (TMCS) were purchased from Lachema (Brno, Czechoslovakia), and TMAA from Koch-Light. All the reagents were ordered in the purest possible form.

The chemicals were mostly stored in a refrigerator (4°) and, except HMDS, which was re-distilled, were used without further treatment.

Apparatus

The reactions were carried out in stoppered 3-ml glass tubes or in 1-ml reaction vessels with Teflon-lined caps (Supelco).

GLC/FID analysis was carried out on a 1.8 m × 3 mm I.D. glass column filled with 5% SE-30 (Varian-Aerograph, Palo Alto, Calif., U.S.A.) on Chromaton N-AW-HMDS (Lachema). The temperature was programmed from 150 to 230° at 5°/min; the detector and inlet temperatures were 240°; the nitrogen carrier gas flow-rate was 25 ml/min; the attenuation was 1/50. The apparatus used was a Chrom 2 chromatograph (Laboratory Equipments, Prague, Czechoslovakia) equipped additionally with a dual FID system and a linear temperature programmer.

GLC/ECD analysis was carried out on a 60 cm × 2 mm I.D. glass column filled with 3% OV-17 on 100–120 mesh Chromosorb W, HP (Supelco). The ECD was fitted with ^{63}Ni foil (20 mCi) and was kept at 240° and 8 V. The inlet temperature was 250°, the column temperature was 170, 190, 210, or 230° (isothermal operation was carried out in order to estimate the relative molar responses to lindane and *p,p'*-DDT) or was programmed from 165 to 215° (or from 150 to 190°) with a gradient of 2°/min (see Fig. 4). The argon–methane (9 : 1) flow-rate was 30 ml/min. The attenuation was 1×10^{-9} A. The chart rate was 30 cm/h.

A Packard Model 7820 chromatograph, with dual argon ionization/electron capture detector was used (Packard, Downers Grove, Ill., U.S.A.). The oven–detector connection was modified for an all-glass system and the electrometer power supply was fitted with a potentiometer for refinement of the voltage setting in the range 0 to25 V.

Procedure

The oxazolidinones were prepared as described in the preceding study[8]. Either the amino acids or their hydrochloride salts were employed. For the GLC/FID studies 1, 2 and 3 μmoles of tyrosine, MIT and DIT, respectively, were taken and then treated with 120 μl of solvent (1:100 dilution of pyridine in acetonitrile) and 30 μl of DCTFA.

The amounts of the three amino acids used for the ECD studies, each treated with 150 μl of solvent and 10 μl of DCTFA, were : 200, 400 and 600 pmoles, respectively. The FID stock solution was used at 1:5000 dilution.

In both cases, the samples were dissolved (max. 5 min) in the medium at room temperature and then heated for 5 min at 50°.

Two methods were employed for the second reaction step. In Method A the phenolic group was esterified directly (the anhydride or silane was added to the first reaction medium) within 15 min reaction time. In Method B the reaction medium was at first evaporated and then the residue was dissolved and esterified for 30 min. The procedures are presented schematically in Table I.

TABLE I

REACTION CONDITIONS AND REAGENTS FOR THE SECOND ACYLATION SILYLATION STEP

Method A	*Method B*
TFAA: plus 10 μl; 20°	30 μl TFAA plus 150 μl benzene; 20°
HFBA: plus 10 μl; 50°	10 (or 20) μl HFBA plus 150 μl acetonitrile; 50°
AA: plus 50 μl; 50°	50 μl AA plus 150 μl acetonitrile–pyridine (100:1); 50°
TMAA: plus 50 μl; 70°	150 μl TMAA plus 50 μl acetonitrile–triethylamine (4:1); 70°
HMDS: plus 50 μl; 60°	50 μl HMDS plus 150 μl acetonitrile–pyridine (100:1); 60°

Afterwards, an aliquot of hexadecane in toluene was added and 1/200 of the volume was injected (FID study, Figs. 1 and 2). If the samples are introduced to ECD analysis, the second reaction medium might be evaporated (except the samples treated with AA and HMDS which could be injected directly) as was in the case of TFAA treatment (Fig. 1c). Before injecting it in the ECD system, the residue was dissolved in ethyl acetate and an aliquot containing lindane and *p,p'*-DDT in toluene as internal standards (molar ratio 1 : 3) was added. Another possibility was to remove the halogenated reagents (HFBA, DCTFA) by extraction (Fig. 1d). After esterification with HFBA (even oxazolidinones esterified with AA were proved if they were not hydrolyzed during extraction) 200 μl of 0.5 *N* HCl and 400 μl of toluene were added and, after shaking the samples for 2–3 min, the water phase was neutralized by 200 μl of 0.5 *N* potassium carbonate.

The simultaneous analysis of monoacylated oxazolidinones and diacylated methyl esters of amino acids (Fig. 3) was performed after preparation of the derivatives according to Method B. Both solutions, oxazolidinones in acetonitrile and methyl esters in methanol, were evaporated in one reaction vial and the dry residue was then treated with the esterification reagent.

RESULTS AND DISCUSSION

The second reaction product in the condensation reaction of amino acid with DCTFA is water. In most esterification reactions (*e.g.*, esterification of amino acid butyl esters with TFAA), strongly anhydrous conditions are required to prevent an easy hydrolysis of the just esterified protonic group. However, in the case of oxazo derivatives, the amino group is firmly bound in a five-membered ring, and so only the hydrolysis of other esterified groups need be considered.

Having compared Methods A and B, the question could be answered if condensation water present in the reaction medium does not decrease the esterification yield. It appeared that direct addition of anhydride (or silane) into the medium of the

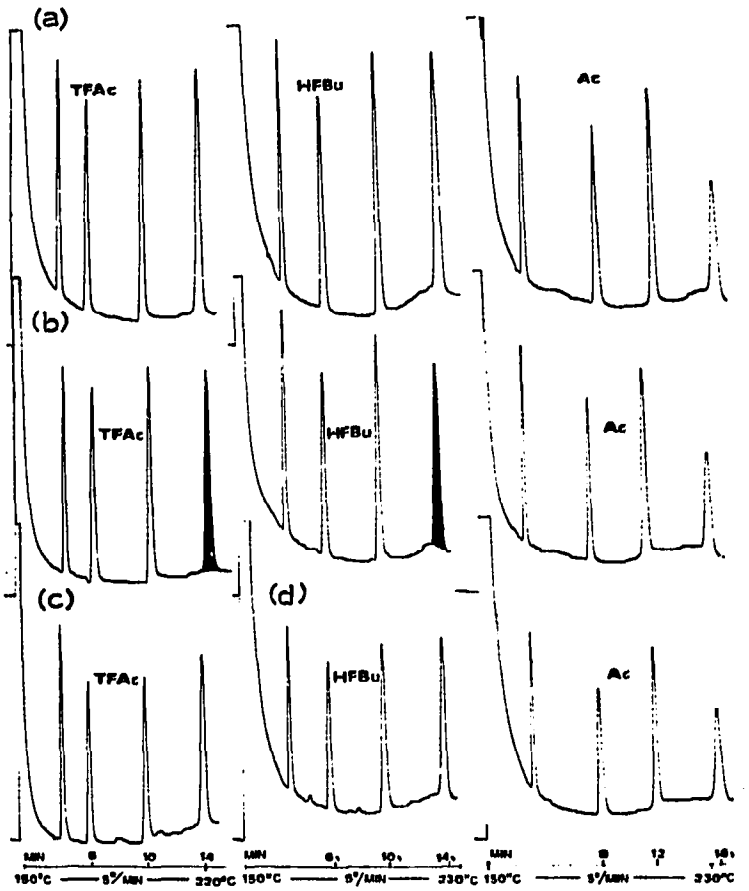


Fig. 1. GLC/FID analysis of O-trifluoroacetyl, heptafluorobutyryl, and acetyl esters of tyrosine (5 nmoles), MIT (10 nmoles), and DIT (15 nmoles) oxazolidinones. The first peak represents hexadecane (2 nmoles). The retention times are shown below the chromatograms. The black peaks represent only partial conversion. (a) Direct acylation; (b) acylation after evaporation; (c) twice evaporated; (d) extraction after acylation. For further explanations, see text.

preceding reaction (Fig. 1a) is more convenient for following esterification than the previous evaporation of reagents from the first reaction step. An excess of DCTFA aids the subsequent dehydration during ester formation, because DCTFA binds all the reaction water (by forming a hydrate). Esterification under these conditions proceeds more easily (required time 15 min) than under anhydrous conditions after previous evaporation of the first reaction medium (30-min esterification). Under these conditions even approx. 5% losses of yields of trifluoroacetyl and heptafluorobutyryl oxazolidinones of diiodotyrosine were observed (Fig. 1b). Even though this phenomenon is obviously accidental (the simultaneous esterification of methyl esters and oxazolidinones according to method B did not confirm any loss of the yield; see Fig. 3), the probability of its occurrence is higher in this case.

The use of AA and HMDS was also successful, even if relatively mild conditions were chosen. The addition of pyridine to AA or TMCS did not improve the yield. If oxazo derivatives and methyl esters were esterified together, the addition of

TMCS even led to a decrease in the yield of the methyl esters. Likewise, the use of stronger silylating agents (*e.g.*, bis(trimethylsilyl)acetamide, BSA) with simultaneous elevation of temperature to 100° for 30 min led rather to a decrease of the yield; partial decomposition of oxazolidinone trimethylsilyl derivatives was observed and so the use of BSA is not recommended. The use of HMDS alone under the conditions described led to yields which could be considered as quantitative.

TMAA was used for esterification of thyroidal amino acid methyl esters in conjunction with triethylamine (TEA)[9]. However, even under these drastic reaction conditions, no quantitative esterification was observed (Fig. 2b). An addition of this reagent directly into the medium of proceeding reaction resulted in formation of unknown peaks (Fig. 2a).

It is well known that halogenated compounds are characterized by lower responses in an FID than compounds containing methylene groups. To find the extent to which the oxazolidine ring contributes to a decrease in FID response, these derivatives were analysed with hexadecane (internal standard) and with esterified methyl esters. The results are shown in Fig. 3. The retention times indicate that there are no significant differences between retention times of N-acyl-carboxymethyl and oxazo compounds and, as in the case of methyl esters, trifluoroacetyl oxazo derivatives are eluted first, whilst trimethylacetyl oxazo esters are eluted last. The relative molar responses (*RMR*) of these two groups are summarized in Table II. Each value (in both Tables II and III) was calculated from five determinations at least. The responses of both groups do not differ very much; however, the fact that the responses of both

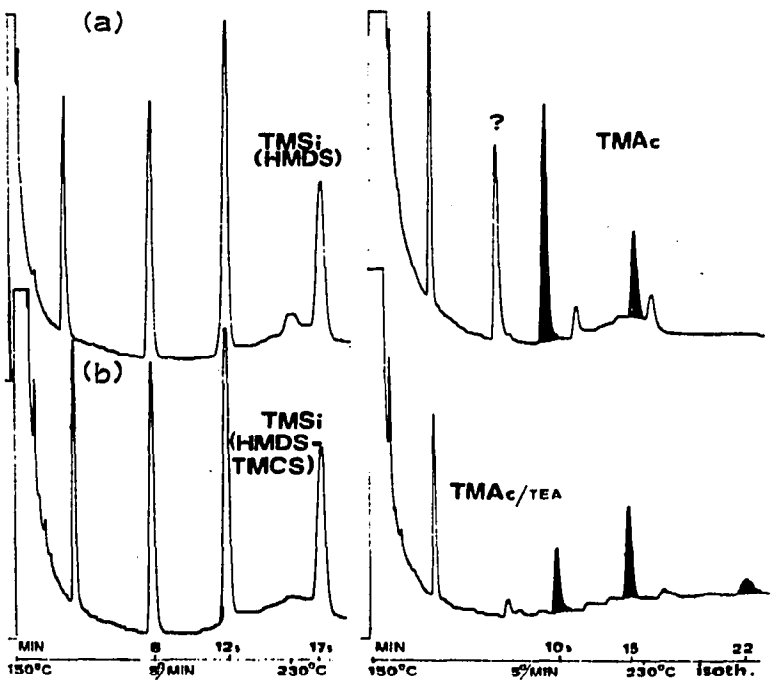


Fig. 2. GLC/FID of O-trimethylsilyl and O-trimetylacetyl esters of three oxazolidinones. (a) Direct derivatization; (b) derivatization after evaporation. For details, see the legend to Fig. 1.

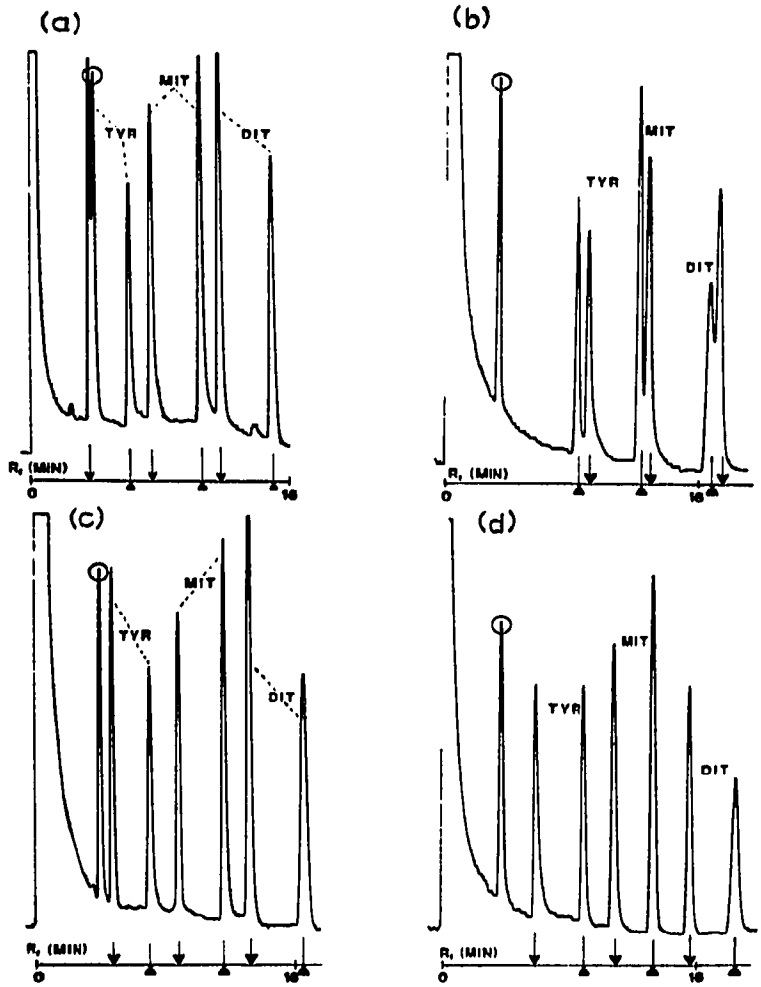


Fig. 3. GLC/FID analysis of O-acyl oxazolidinones and N,O-diacyl methyl esters of tyrosine, MIT and DIT: (a) trifluoroacetyl, (b) acetyl, (c) heptafluorobutyryl, and (d) trimethylsilyl. The amounts injected are the same as in Fig. 1. For the retention times, see the horizontal axis. For the GLC conditions, see the text. Th *RMR* values are shown in Table II. ○, Hexadecane (internal standard); ▼, N,O-diacyl methyl ester; ▲, O-acyl oxazolidinone.

halogenated homologues of tyrosine are not decreased at the same rate is remarkale. Whilst in the case of oxazolidinones almost regular decreases in direction from tyrosine to DIT occur, with methyl esters there is a visible difference between tyrosine and MIT response, where as the DIT response does not differ significantly from that of MIT.

The findings obtained by GLC/FID studies were further applied to analysis with an ECD. The recent development of electron capture detection as well as the appearance of a new type of linear ECD[10] invokes the question of preparation of convenient halogenated derivatives. The problem is not only in the derivatization itself, but also in the removing of halogenating reagent from the sample before analysis.

The most general way is evaporation of the reagent. DCTFA (b.p. 45°) and

TABLE II

RELATIVE FID MOLAR RESPONSES OF O-ACYL(SILYL) OXAZOLIDINONES AND N,O-DIACYL(SILYL) METHYL ESTERS OF TYROSINE, MIT AND DIT TO HEXADECANE AND TO DERIVATIZED TYROSINE

Type of ester	$RMR_{tyrosine}$		$RMR_{hexadecane}$	
	Oxazo	*Methyl*	*Oxazo*	*Methyl*
Trifluoroacetyl				
Tyrosine	1.00	1.00	0.41	0.43
MIT	0.70	0.56	0.29	0.24
DIT	0.50	0.50	0.21	0.22
Heptafluorobutyryl				
Tyrosine	1.00	1.00	0.47	0.50
MIT	0.70	0.60	0.33	0.30
DIT	0.51	0.55	0.24	0.27
Acetyl				
Tyrosine	1.00	1.00	0.47	0.45
MIT	0.69	0.71	0.32	0.32
DIT	0.41	0.65	0.19	0.29
Trimethylsilyl				
Tyrosine	1.00	1.00	0.51	0.47
MIT	0.68	0.58	0.35	0.27
DIT	0.40	0.43	0.20	0.20

TFAA (b.p. 39°) could be easily evaporated, even if the anhydride was added directly to the medium or after the evaporation of the reaction mixture. However, the problem is in the stability of the resulting trifluoroacetyl esters. Änggård and Sedvall[11] found that simple evaporation of the reagent under nitrogen led to almost 50% decomposition of trifluoroacetylated metabolites of catecholamines. Similarly, the analysis of N,O-bis(trifluoroacetyl) methyl esters of T_3 and T_4 could not be reproduced[12]. The most important problem was that the trifluoroacetyl ester of a hydroxy group is easily hydrolysed. This difficulty does not occur with oxazolidinones, in which the esterified phenolic group does not undergo such easy hydrolysis, and so trifluoroacetyl oxazolidinones could well be analysed even after two-fold evaporation of reagents (Figs. 1c and 4). It was satisfactory to find a high response in ECD (Table III) and easy preparation of these derivatives.

However, if HFBA is added to the reaction medium of the first reaction, the

TABLE III

RELATIVE ECD MOLAR RESPONSES (*RMR*) OF O-ACYL OXAZOLIDINONES AND N,O-BIS(HEPTAFLUOROBUTYRYL) METHYL ESTERS OF TYROSINE, MIT AND DIT TO LINDANE

The *RMR* of *p,p'*-DDT to lindane under the conditions described was 0.44.

Relative molar response to lindane	*Oxazolidinone*			*Methyl ester*
	Trifluoroacetyl	*Heptafluorobutyryl*	*Acetyl*	*Heptafluorobutyryl*
Tyrosine	0.84	1.03	—	1.11
MIT	0.90	1.18	0.84	1.33
DIT	0.79	0.94	0.70	1.17

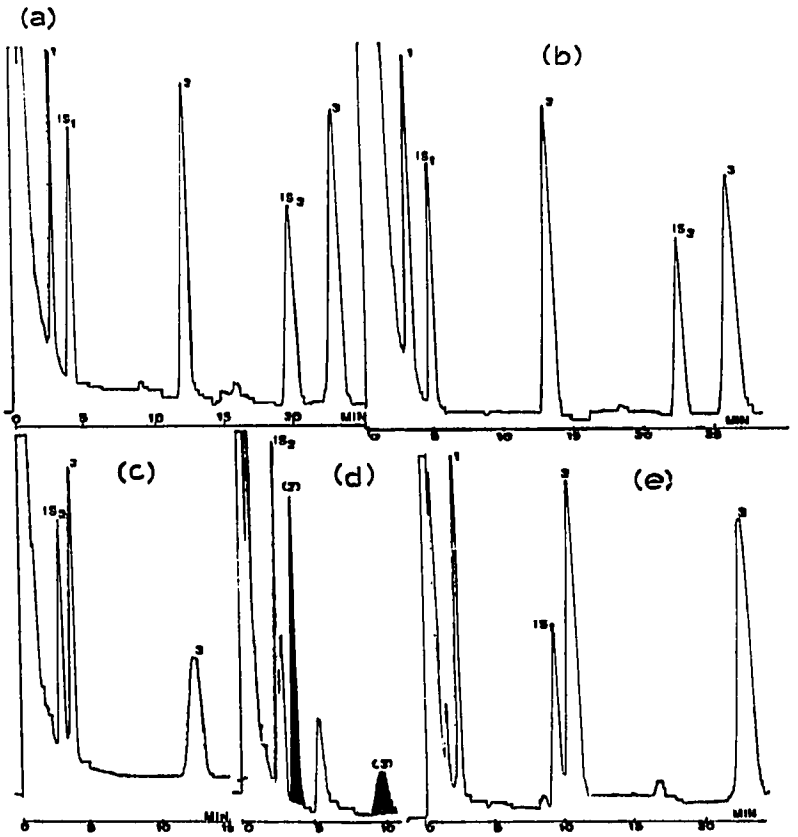


Fig. 4. GLC/ECD analysis of 1, 2 and 3 pmoles, respectively, of tyrosine (1), MIT (2), and DIT (3) oxazolidinone O-acyl esters: (a) trifluoroacetyl, (b) heptafluorobutyryl, (c) acetyl, and (d) trimethylsilyl. For comparison, the N,O-bis(heptafluorobutyryl) esters of these three acids were also chromatographed (e). The following two internal standards were used: IS_1 = lindane (1 pmole) and IS_2 = *p,p'*-DDT (3 pmoles). The *RMR* values to lindane are shown in Table III. Temperatures: (a) 165–210°, 2°/min; (b) 160–210°, 2°/min; (c) 230°, isothermal; (d) 235°, isothermal; (e) 150–190°, 2°/min.

pyridine present (1 % solution in acetonitrile) binds a fraction of this anhydride as an oil drop on the bottom of the tube. This oil could not be removed even by several hours' evaporation under nitrogen at 50° and so estimation by ECD was impossible. Therefore the earlier reports recommending acidic[13] or basic[14] extraction were examined. In this way, an excess of HFBA is removed by decomposition and heptafluorobutyryl esters could be extracted into an organic phase. This technique appeared to be suitable for extraction of heptafluorobutyryl and acetyl oxazolidinones, as is demonstrated by the peaks in Fig. 1d. However, in the picogram range of ECD analysis the extraction failed and, therefore, the "two-evaporation" technique (as in the case of trifluoroacetyl esters; Fig. 1c) was chosen for the estimation of heptafluorobutyryl oxazolidinones, prepared according to Method B.

Thus, the remaining problem is in the extraction of picogram amounts of heptafluorobutyryl oxazolidinones. This will be discussed later in connection with analysis of thyroidal hormones.

If AA or HMDS were used as esterifying agents (preceded by evaporation of the reaction medium of the first reaction), the sample could be applied directly to ECD analysis. As demonstrated in Fig. 4, the same quantitative results as by FID analysis were obtained if AA was used. The use of HMDS at picogram concentrations of sample did not bring the expected results. The problem of a convenient silylating reagent is yet to be studied in connection with the analysis of endocrinologically important amino acids.

The ECD response of oxazo derivatives was estimated by determination of *RMR* values with lindane as internal standard and by comparison with the response of N,O-heptafluorobutyryl methyl esters (Table III). These values were determined in both isothermal as well as in temperature-programmed operations (Fig. 4) and the eventual change of response (caused by temperature programming) was detected by *p,p'*-DDT as second internal standard. When compared with isothermal operations, the deviations found were approx. 5%. The *RMR* values shown in Table III are in accordance with an assumption that the oxazolidine ring, carrying two chlorine and four fluorine atoms, belongs to compounds with high ECD response. This response is so marked that other halogen atoms present in the molecule, as well as esterification with various halogen-containing anhydrides, could not affect it significantly (see, *e.g.*, differences between tyrosine, MIT and DIT response or between trifluoroacetyl and heptafluorobutyryl oxazolidinone response, respectively). The O-heptafluorobutyryl oxazolidinone response is close to that of N,O-bis(heptafluorobutyryl) methyl esters, which represent one of the most marked responses among known ECD derivatives.

A great advantage of the above described oxazo derivatives of amino acids is their stability. In organic solvents (*e.g.*, ethyl acetate or benzene) the oxazolidinones could be stored for several months, their acetyl and heptafluorobutyryl esters are stable for several weeks and their trifluoroacetyl (or trimethylsilyl) esters did not undergo decomposition for several days, when stored at 4°.

Therefore, the application of oxazolidinones as excellent derivatives for the analysis of biochemically important amino acids seems to be reasonable.

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