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USE OF BORANE AS REDUCING AGENT IN SEQUENCE ANALYSIS OF PEPTIDES BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

The properties of borane as a reducing agent for N-acylated peptides, as compared to the known lithium aluminium deuteride procedure have been investigated. The resulting polyamino-alcohols are converted into the corresponding trimethylsilyl ethers and submitted to capillary gas chromatography. With borane the esterification of carboxyl groups is not required, reduction proceeds very fast and work-up of the reaction mixture is simple. Correspondingly complete derivatization is carried out in a significantly shorter time than with lithium aluminum deuteride. In addition, fewer side-products are formed, even if a very large excess of borane is used. No problems arise with labile peptides, and the yields are high enough to allow derivatization, separation and identification of peptides in amounts as low as 200 pmoles.

The separation of the polyamino-alcohols by capillary gas chromatography was demonstrated, and proved to be superior to the packed columns used hitherto.

INTRODUCTION

The reduction of oligopeptides to the more volatile polyamino-alcohols[1] with lithium aluminium hydride was first used by Biemann *et al.*[2,3] for sequence analysis of peptides by mass spectrometry (MS). The fragmentation pattern in most cases is simple, and easily interpreted, and yields sufficient information for the determination of the structure of the parent peptide. The procedure was later applied by Nau and coworkers[4–7] to N-pentafluoropropionyl and N-heptafluorobutyryl peptides, generating the most volatile peptide derivatives hitherto known. As a consequence of higher volatility, longer oligopeptides are amenable to gas chromatography (GC), whereby unambiguous sequence determination of a protein is facilitated[8]. Along with their favorable GC–MS properties, these derivatives exhibit predictable retention indices, offering the possibility of cross-checking the data obtained from MS[9,10].

Nevertheless this method has not yet found widespread application for sequence determination of proteins, because relatively large amounts of every parent

peptide are necessary for complete derivatization and GC–MS analysis[9]. Assuming an optimal cleavage by partial hydrolysis into overlapping oligopeptides, at least 10 nmoles of the peptide or protein to be sequenced is required, whereas with modern Edman degradation, sequencing at the subnanomole level is feasible[11]. Also, until now only packed columns have been used for the separation of the amino-alcohols.

The sensitivity of sequence analysis by GC–MS can be improved along two lines: less destructive derivatization and more efficient separation of the constituents of the oligopeptide mixture.

The objective of this contribution is the application of capillary columns and the investigation of scope and yield of the reduction with borane as compared to lithium alumnium deuteride (LAD). Among the reagents capable of effecting the reduction of carbonyl groups to methylene groups, borane was shown to react rapidly with high yields[12]. Tertiary and secondary amides are reduced to the corresponding amines, and carboxyl groups are reduced as such without the necessity of previous esterification. C–N bond cleavage, sometimes observed with LAD, does not take place with borane[13]. Moreover, the reduction of fluoroacetamide derivatives, where the action of LAD may cause hydrogenolysis of C–F bonds[14], can be successfully carried out with borane[15]. The chemical properties of borane have been used to reduce selectively amide linkages in peptide methyl esters[16,17] or carboxyl groups in peptides[18–20]. However, the use of borane for GC–MS analysis of peptides has not been tried until recently[21].

EXPERIMENTAL

GC was carried out with a Carlo Erba Fractovap 2101 gas chromatograph equipped with an SE-52 capillary column, 19 m × 0.3 mm I.D.; carrier gas hydrogen 0.25 atm; split ratio 1:25; injector and flame ionization detector temperature, 300°; temperature programme: 120° isothermal for 2 min, heating rate 4°/min, final temperature 220°. MS was carried out with an LKB 9000.

A stock solution of 10 mg (53 μmoles) of N-acetylalanylglycine, 11 mg (43 μmoles) of N-acetylprolylserine, 10 mg (31 μmoles) N-acetylaspartylphenylalanine and 10 mg (26 μmoles) N-trifluoroacetylaspartylphenylalanine in 100 ml absolute methanol was prepared.

Hexadeuterodiborane was prepared from boron trifluoride and LAD as described in the literature[22].

Lithium aluminium deuteride reduction

The appropriate volume of peptide solution was placed in a screw-cap derivatization flask and treated with excess diazomethane at room temperature for 10 min. The solvent was evaporated in a gentle stream of nitrogen. An appropriate volume of 1.8 *M* solution of LAD in tetrahydrofuran (THF) was added, and the flask closed and heated at 90° for 24 h. The flask was then cooled to 0°, the contents were diluted with an equal volume of diethyl ether and the excess LAD was destroyed by careful addition of water-saturated ethyl acetate followed by addition of a drop of water. A fine precipitate of aluminum hydroxide immediately formed. After centrifugation the solution was extracted three times with methanol. The extracts were combined, the solvent was evaporated and the residue extracted with chloroform. The solvent

was evaporated under a stream of nitrogen and the residue treated with 40 μl pyridine and 20 μl trimethylsilyldiethylamine for 30 min at 80°. After cooling this solution was used for gas chromatography.

Borane reduction

The appropriate volume of peptide solution was placed in a screw-cap derivatization flask, the solvent evaporated under a gentle stream of nitrogen and the residue dissolved in the appropriate volume of a 1 *M* solution of trideuterioborane in THF. The flask was closed and heated to 90°. After 30 min the solution was cooled to 0° and the excess of borane destroyed by careful addition of methanol until no more hydrogen was evolved. The solvent was evaporated, the residue dissolved in 100 μl of 1 *N* hydrogen chloride in absolute methanol, the flask closed and heated for 30 min at 90°. This operation was repeated. After evaporation of solvent the residue was dissolved in 200 μl of a 25% potassium carbonate solution and extracted three times with 200 μl dichloromethane each time. The extracts were combined, the solvent was evaporated and the residue treated with trimethylsilyldiethylamine as described above.

RESULTS AND DISCUSSION

A series of experiments with LAD and borane were run with a constant volume (1 ml) of the stock solution of the four N-acyl peptides. The reduction of the peptides contained in 1 ml of stock solution requires a stoichiometric amount of 25 μequiv. of hydride for borane or 16 μequiv. for LAD.

Reduction was effected with various volumes of solutions of each reagent in THF. For each experiment the ratio of the added amount of hydride to the theoretically required amount was calculated and related to the corresponding peak heights for each peptide. The values obtained are shown in Fig. 1, which reveals an obvious dependence of the yield of polyamino-alcohols on this ratio. A maximum yield is obtained with LAD at a ratio of 10:1. The yield decreases rapidly with increasing amounts of LAD. The chromatograms showed that the decrease is intimately associated with an increasing complexity of the mixture, which becomes enriched in

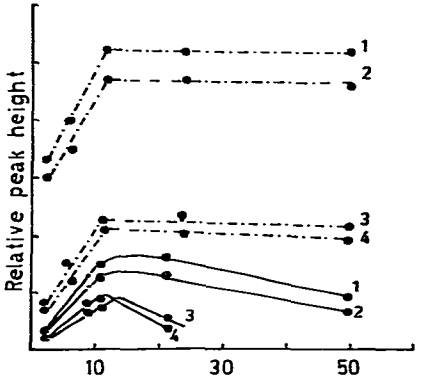

Fig. 1. Relative yields of polyamino-alcohols in relation to the ratio between the added amount of hydride and the theoretically required amount. ———, $LiAlD_4$; ·–·–·, BD_3. 1 = N-Ac-Ala-Gly, 2 = N-Ac-Pro-Ser, 3 = N-TFA-Asp-Phe, 4 = N-Ac-Asp-Phe.

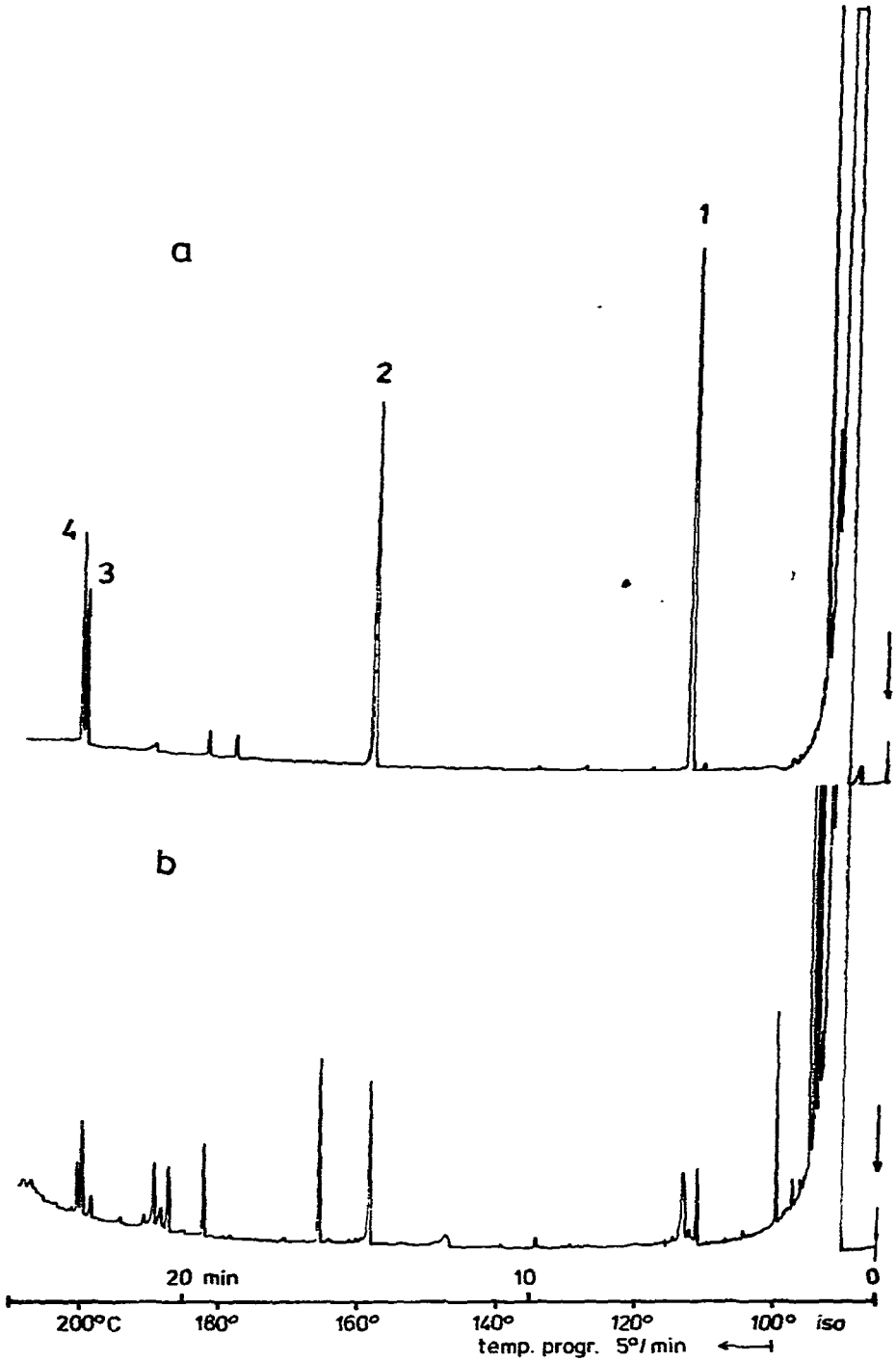

Fig. 2. Capillary gas chromatography of O-trimethylsilylated diamino-alcohols obtained from reduction of 1 ml of peptide stock solution with (a) 400 μl borane and (b) 100 μl lithium aluminum hydride in THF; the hydride ratio was 45:1 in both experiments. Chromatographic conditions as given under Experimental. Injected aliquot 1/120 (a), 1/60 (b): attenuation of the electrometer response 1/32. In order to avoid complete destruction of peptides 3 and 4, reaction time with lithium aluminum deuteride was decreased from 24 to 14 h. 1 = N-Ac-Ala-Gly; 2 = N-Ac-Pro-Ser; 3 = N-TFA-Asp-Phe; 4 = N-Ac-Asp-Phe.

secondary reaction products (Fig. 2b). The aspartyl phenylalanine derivatives disappear especially rapidly, which may be due to destruction of the peptide by excess LAD, racemization at the asymmetrical carbon under the prolonged treatment at high temperature resulting in diastereomers of different retention time, or adsorption of the polyamino-alcohols on the precipitate of highly active aluminium hydroxide. The reaction with borane shows a quite different course. The yields of polyamino-alcohols increase with increasing molar ratio to a maximum value which is not significantly affected by the presence of a large excess of borane. No significant formation of secondary products is detected in the chromatograms (Fig. 2a).

In another series the minimum detectable amount of peptide has been determined for both reagents (Table I). Various volumes of the peptide stock solution (0.5, 1.0, 5, 10, 20 μl) were reduced with 1 μl of the corresponding reagent solution. This is regarded as minimum volume because traces of water present in the sample and absorbed on glass surfaces unpredictably reduce the amount of hydride available for reduction. It is clearly shown that the sample size amenable to analysis is at least 10 times smaller with the borane technique than with LAD.

TABLE I

REDUCTION YIELDS OF VARIOUS AMOUNTS OF DIPEPTIDES WITH 1 μl 1 *M* BD_3 IN THF AND 1 μl 1.8 *M* $LiAlD_4$ IN THF, RESPECTIVELY

Each 1 μl of peptide stock solution contains 0.53 nmoles N-Ac-Ala-Gly, 0.43 nmoles N-Ac-Pro-Ser, 0.31 nmoles N-Ac-Asp-Phe and 0.26 nmoles N-TFA-Asp-Phe. 80% of the final solution of the TMS ethers of diamino-alcohols was injected for GC (for conditions see Experimental).

Volume of stock solution (μl)	*Reagent*	*Relative peak heights (arbitrary units)*			
		N-Ac-Ala-Gly	*N-Ac-Pro-Ser*	*N-TFA-Asp-Phe*	*N-Ac-Asp-Phe*
0.5	1 μl BD_3	2	2	1.2	1
1		5.2	2.3	1.7	1.65
5	1 μl $LiAlD_4$	1	1.3	—	—
10		2.5	2.5	—	—
20		5.6	3.8	2.2	2.7

With borane we were able to derivatize and detect amounts of as little as 0.2 nmoles, even in the case of the labile aspartylphenylalanine derivatives. With LAD the lower limit of detection was in the range 2–5 nmoles, which is in agreement with the findings of others[9].

The reduction with borane proved to be superior in other respects. For N-acetylprolylserine the loss of the N-terminal blocking group, as reported for the LAD procedure[10], does not occur.

An important feature from a practical point of view is the decrease in reaction time. The derivatization of N-acylated peptides is completed in less than 3 h, compared with the 24 h required by the LAD procedure. Esters, which may be present if carboxyl groups have to be esterified in order to increase the solubility of acidic peptides, are also reduced by borane under the adopted conditions. The precalculation of the correct amount of reducing agent is unnecessary, as large excesses of

borane do not affect the yield of reaction significantly. Determination of the correct ratio of reducing agent to reducible groups is necessarily inaccurate, especially with very small samples.

The possibility of hydroboration of aromatic and heterocyclic amino acids was investigated by MS. As expected, no evidence for hydroboration of amino acids containing an aromatic double-bond system (phenylalanine, tyrosine, tryptophan) was found. The direct-inlet mass spectrum of N-trifluoroacetylhistidine revealed no product of such a side-reaction of the imidazole ring. We believe that the reported procedure will raise the sensitivity of sequence analysis of peptides by GC–MS by one order of magnitude, thus rendering this procedure more compatible for combination with ultrasensitive methods involving high-performance liquid chromatography[23]. As mentioned above, application of capillaries instead of packed columns will further lower the detection limits, and if splitless injection were employed (which has not been the case in this study), sample sizes in the lower pmole range would be amenable to analysis by GC–MS.

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