A Novel Approach for the Quantitative Study of Reductions by Metallic Sodium in Liquid Ammonia. Cleavage of Protecting Groups from Protected Oxytocein

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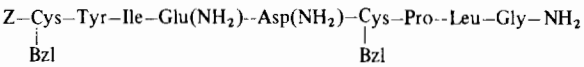
Abstract

A novel method has been developed for the quantitative characterization of reductions with metallic sodium in liquid ammonia. The changes in redox potential and conductivity of solutions were measured by 'titration' in a glass apparatus. The titration curves obtained provided information on the stoichiometry of the reactions. The intermediates and products were analyzed by means of HPLC. The studied model reaction was the cleavage of the protecting groups of protected oxytocein, among others, via study of the behaviour of smaller peptides and model substances.

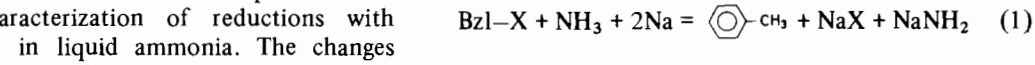
Introduction

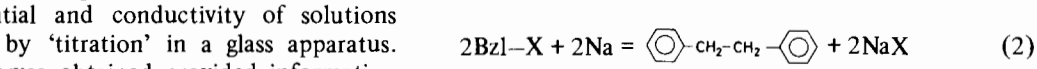
Reduction with metallic sodium in liquid ammonia has long been used in peptide chemistry to cleave benzyl-type and tosyl protecting groups [1]. The application of this method is based on empirical prescriptions. The mechanisms and stoichiometry of the reactions are only partly known. In order to obtain more exact information on the processes, we have developed a method for the study of such reactions via quantitative 'titration'. Our primary aim was the quantitative investigation of the cleavage of protecting groups from protected oxytocein.

In our case protected oxytocein contained two benzyl (Bzl) and one benzyloxycarbonyl (Z) protecting groups



The literature data [2, 3] indicate that the benzyl groups can be cleaved off theoretically in two routes: reduction with two moles of sodium leads to the formation of toluene, while with one mole of sodium the product is diphenylethane [4, 5]:





The factors determining the ratio of the two pathways are unknown.

Since protected oxytocein is a multifunctional molecule, some model substances were used to facilitate the understanding of its chemical behaviour. The reduction of the following substances was performed: triglycine, *N*-acetyl-triglycine, benzyl benzoate, ethyl *S*-benzylthioglycolate (ESBT) and ethyl thioglycolate. The results of these measurements are reported in this paper.

Experimental

Apparatus

A glass apparatus was constructed (Fig. 1) for the quantitative characterization of reductions with metallic sodium in liquid ammonia by 'titration'. In the course of the procedure, the temperature, the conductivity and the redox potential of the solutions were measured.

In Fig. 1, vessel I is the burette and vessel II the measuring cell. Both vessels are marked with calibrated volumetric scales. In vessel I the temperature (3) and redox potential (1)** were measured; in vessel II, the temperature (3), change in potential (1) and change in conductivity (2)† were measured.

The temperature was measured with a thermistor connected to a D1216 digital multimeter (Norma

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**The latter serves for control of the stability of the standard sodium solution.

†The two latter changes reflect the course of the reactions.

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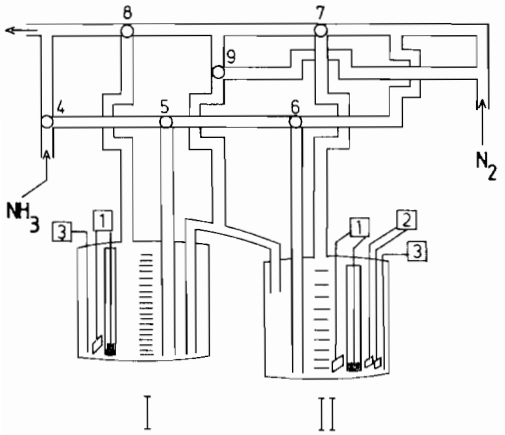


Fig. 1. Apparatus for 'titration-type' study of reduction reactions by metallic sodium in liquid ammonia. I, Burette; II, measuring cell. 1, Platinum-reference electrode pair; 2, two platinum electrodes; 3, thermistor; 4–9, Taps.

Messtechnik, F.R.G.). Conductivity was measured with an OK 102/1 Conductometer (Radelkis, Hungary), with two Pt electrodes. The redox potential was measured with a PHM 52b digital pH-meter (Radiometer, Denmark). A platinum electrode served as indicator electrode, while the reference electrode was $Ag/KCl_{(sat)}-AgCl_{(sat)}$ in liquid NH_3 [6]. Both electrodes were home made.

The condensation of ammonia and cooling of solutions were achieved with mixtures of acetone and dry ice, the appropriate temperature being attained by bubbling nitrogen through the solution.

Measurements

The apparatus was dried at 403 K for 3 h. Titrations were carried out in an atmosphere of nitrogen, dried by passage through sodium-asbestos and solid NaOH. The bubbling of nitrogen through the solution also affected the mixing of the solution. Ammonia was purified by distillation from sodium [7, 8].

The substance to be titrated was weighed with analytical accuracy into vessel II, and the necessary quantity of ammonia was then condensed on top of it. Ammonia was also condensed into burette vessel I and solid sodium was dissolved in it. Ammonia was likewise introduced by condensation into the reference electrodes. After this procedure the volume of liquid ammonia in the two cells was set to appropriate values. Before titration, solutions were mixed for 30 min by the bubbling of nitrogen. The aliquot parts of standard sodium solution were pushed into the measuring cell by a nitrogen flow. After the addition of each aliquot, the temperature of both solutions was set to 228 K and the changes in volume, conductivity and redox potential (e.m.f.) were recorded.

HPLC Separation of Reaction Products

Experiments were carried out with LKB 2150 (LKB, Bromma, Sweden) and Liquochrom OE (Labor MIM, Hungary) liquid chromatographs, equipped with prepacked Hypersyl ODS columns, 10 μm, 250 × 4.6 mm I.D. (Bio Separation Technology, Budapest, Hungary).

Analysis of triglycine and N-acetyl-triglycine

Eluent: acetonitrile:0.01 M KH_2PO_4 (pH = 2.2) = 3:97; containing 0.2 M $NaClO_4$. Detection: UV, 220 nm.

Analysis of toluene, diphenylethane, benzyl-benzoate and their reduction products

Eluent: methanol:water = 80:20. Detection: UV, 254 nm.

Analysis of oxytocein and oxytocin [9–12]

Eluent: acetonitrile:0.01 M KH_2PO_4 = 25.75, containing 0.1 M $NaClO_4$. Detection: UV, 220 nm.

Materials

Protected oxytocein and oxytocin were products of the Chemical Works of Gedeon Richter (Budapest, Hungary); benzyl benzoate and ethyl thioglycolate were purchased from Fluka (Buchs, Switzerland); triglycine from Reanal (Budapest, Hungary); *N*-acetyl-triglycine, ethyl *S*-benzyl-thioglycolate (ESBT) and diphenylethane were prepared by usual methods. The oxytocin content of the oxytocin sample was determined by UV spectrophotometry, SERVA p.a. tyrosine being used for the calibration.

The organic solvents used were of HPLC grade (E. Merck, Darmstadt, F.R.G.). Buffers and salt solutions were made from Merck, BDH (Poole, U.K.) and Reanal (Budapest, Hungary) reagents, using water doubly distilled from glass apparatus.

Results and Discussion

Triglycine served first for modelling the reduction of peptide molecules with sodium in liquid ammonia. This compound reacted with 3 equivalents of sodium (Fig. 2). The first step of titration involves the reduction of the NH_4^+ ion originating from ionization of the terminal carboxyl group (which has a strongly acidic character in liquid ammonia).

$$NH_4^+ + Na \longrightarrow NH_3 + \tfrac{1}{2}H_2 + Na^+ \qquad (3)$$

The mobilities of NH_4^+ and Na^+ ions are nearly equal [13], and the greater degree of association of the sodium salt formed than that of the ammonium one decreases the conductivity. After the first equivalence point, 2 further equivalents of sodium are consumed by the system. To determine whether protons of the peptide bonds or the primary amino

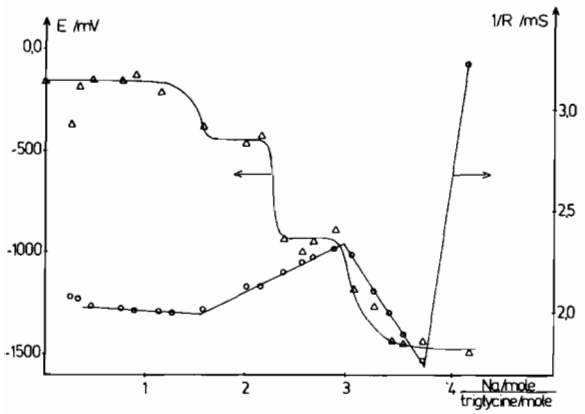


Fig. 2. Potentiometric (△) and conductometric (○) titration curves of triglycine. Concentration of triglycine: 8.5×10^{-3} mol dm^{-3}.

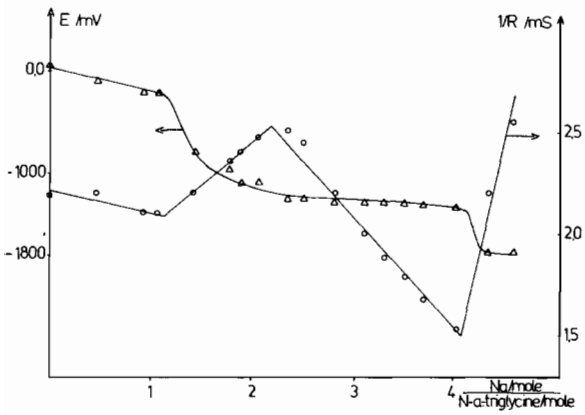


Fig. 3. Potentiometric (△) and conductometric (○) titration curves of *N*-acetyl-triglycine. Concentration of *N*-acetyl-triglycine: 6.3×10^{-3} mol dm^{-3}.

group react in the latter two steps, triglycine with its terminal amino group protected by an acetyl group (*N*-acetyl-triglycine) was examined.

The first reduction step of *N*-acetyl-triglycine can be assigned to the reaction of the NH_4^+ ion originating from ionization of the terminal carboxyl group (Fig. 3). The further 3 equivalents of sodium consumed presumably react with amide protons (two peptide-NH and one acetyl amide-NH), since the molecule does not contain other reducible protons. The conductivity curve shows that the sodium salt formed in the reaction with the second equivalent of sodium is still in dissociated form. The further reduction of the peptide leads to anions having higher negative charges; these are therefore associated with sodium ions in the solution, which results in a conductivity decrease. The end-point of the total reduction process is characterized by a redox potential change and by a strong increase in conductivity due to the appearance of free electrons in the solution.

The behaviour of *N*-acetyl-triglycine suggests that in the reduction of triglycine (Fig. 2) the second and third equivalents of sodium react with the weakly acidic peptide hydrogens. The salt formation in the latter process results first in the conductivity increase and then in its decrease due to the change in association between deprotonated peptides and sodium ions. The corresponding e.m.f. curve shows that the two peptide hydrogen atoms are reduced in two successive steps, because of the different chemical surroundings of the two peptide bonds.

Titration curve of *N*-acetyl-triglycine (Fig. 3) shows that the introduction of a third carboxamide group with acetylation of the α-amino group obliterates the successiveness in the reduction of the three protons. Literature data concerning the reversible deprotonation of amide-*H* by sodium in liquid ammonia are contradictory [14]. In one case the removal of a substituted amide proton was proved by successive *N*-benzylation [15].

The products of the above reactions were analyzed by HPLC to examine whether the reduction with sodium resulted in the cleavage of peptide bonds or not. The HPLC results showed that dissolution of the reduction products of triglycine in water led to the almost complete reestablishment of triglycine, indicating that the reduction did not cause the cleavage of peptide bonds. The amount of minor byproducts formed was below 10%.

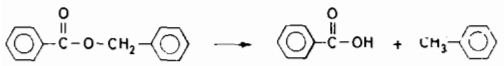
In the analogous process when *N*-acetyl-triglycine was titrated with sodium to different sodium/peptide molar ratios, the amount of reestablished *N*-acetyl-triglycine after dissolution of the reduction products in water was 70–75% (Table 1). The extent of cleavage of the protecting acetyl group from the terminal amino group of the peptide was about 10%, and the extent of other side-reactions was about 15%.

Experiments with triglycine and *N*-acetyl-triglycine clearly show that peptide bonds consume sodium in liquid ammonia. Though the majority of peptides can be regenerated, the significant losses of 10 to 30% refer to the harmful effects of sodium excess applied in the sodium–liquid ammonia deprotection of synthetic peptides [4, 5].

We attempted to use the reduction of *benzyl benzoate* with sodium to model the splitting-off of the benzyl protecting group. Unfortunately, the titration curves of this compound could not be evaluated because it did not dissolve in liquid ammonia; it reacted with sodium in a heterogeneous process, indicated by the change in colour of the solution. HPLC analysis of the products formed in this reaction showed that benzyl benzoate decomposed totally. The peaks in the chromatogram could be assigned to benzoic acid, toluene, benzaldehyde and benzyl alcohol. The first two are the dominating products, so the pathway of the reaction seems to be the following:

TABLE 1. Material Balance for the Reduction of *N*-Acetyl-triglycine

Concentration of protected peptide (mol dm^{-3})	Molar ratio of sodium:peptide	Reestablished protected peptide (mol dm^{-3})	Reestablshed triglycine (mol dm^{-3})	Recovered total triglycine (protected + non-protected) (%)
3.45×10^{-3}	3.66	2.48×10^{-3}	0.3×10^{-3}	81
3.40×10^{-3}	4.71	2.50×10^{-3}	0.4×10^{-3}	86
3.48×10^{-3}	5.07	2.59×10^{-3}	0.5×10^{-3}	84



In liquid ammonia, esters may undergo ammonolysis to yield amide and alcohol [16]. This process is usually catalyzed by ammonium salts. Our HPLC measurements did not reveal any products of ammonolysis of benzyl benzoate in the course of the reaction, even if it was carried out in the presence of ammonium chloride.

Ethyl S-benzylthioglycolate served as a model compound for study of the reductive cleavage of the protecting group coupled to the sulphydryl sulphur. Only a single equivalence point was observed in both the potentiometric and the conductometric curves of the titration after the addition of one equivalent of sodium (Fig. 4). The system consumed additional sodium after the equivalence point. A conductivity increase indicating the appearance of solvated electrons was not observed. This may indicate the catalytic effect of the reduction product(s) on the formation of sodium amide ($Na + NH_3 \rightarrow Na^+ + NH_2^- + \frac{1}{2}H_2$).

This concept is supported by the reactions of *ethyl thioglycolate* with sodium (Fig. 5). The titration curves exhibit one equivalence point, after the addition of one equivalent of sodium, which corresponds to the titration of the proton of the thioalcohol group. Although the product does not contain further groups reducible with sodium, the conductivity increase characteristic of solvated electrons did not appear. This shows that the $^-S–CH_2COOEt$ ion catalyzes the above reaction. This ion forms also in the reaction of ethyl *S*-benzylthioglycolate with metallic sodium.

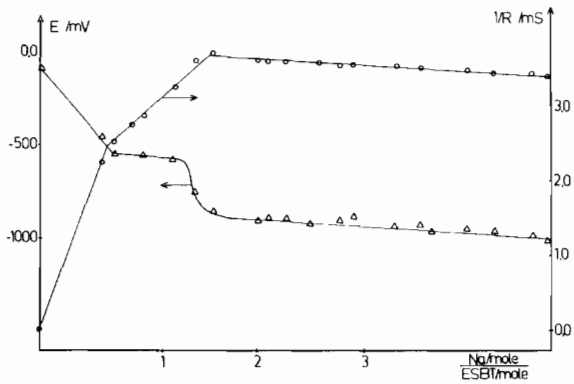


Fig. 4. Potentiometric (△) and conductometric (○) titration curves of ethyl *S*-benzyl-thioglycolate (ESBT). Concentration of ESBT: 9.2×10^{-3} mol dm^{-3}.

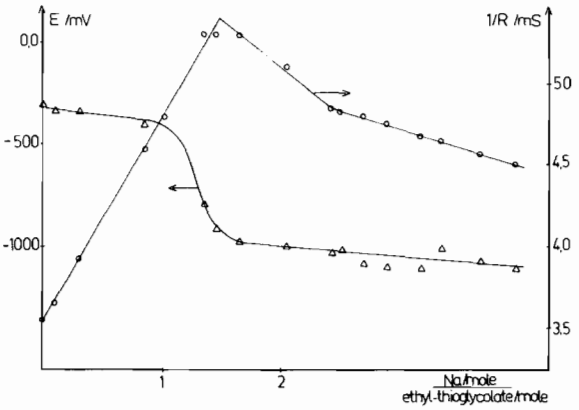


Fig. 5. Potentiometric (△) and conductometric (○) titration curves of ethyl-thioglycolate. Concentration of ethyl thioglycolate: 1.1×10^{-2} mol dm^{-3}.

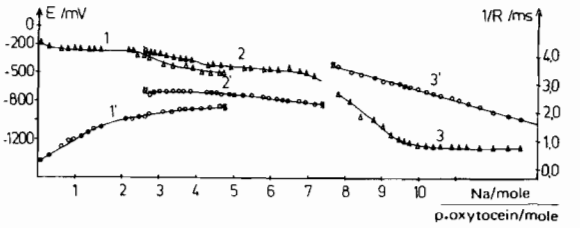


Fig. 6. Potentiometric (1, 2, 3) and conductometric (1′, 2′, 3′) titration curves of protected oxytocein. Concentration of protected oxytocein: 6.0×10^{-3} mol dm^{-3}.

Titration of Protected Oxytocein

Because of the small volume of our burette (vessel I in Fig. 1), the titration curves of protected oxytocein (consumption of 13 equivalents of sodium) were recorded in three successive experiments. Figure 6 shows the potentiometric (1, 2, 3) and conductometric (1′, 2′, 3′) titration curves of protected oxytocein with standard sodium solution. To determine the reduction products, the reaction was stopped at different sodium/peptide molar ratios, the liquid solvent was removed by evaporation,

and the solid product was dissolved in water and, after suitable oxidation, analyzed by HPLC.

The reduction of protected oxytocein with sodium starts with the cleavage of the protecting benzyl and benzyloxycarbonyl groups from the molecule, which consumes 6 equivalents of sodium. In this reaction, mainly toluene is formed. The amount of diphenylethane is negligible. In the subsequent reaction steps, sodium reacts mainly with peptide hydrogen atoms. Certain species formed in the reductions are negatively charged. As the reaction progresses, this charge gradually increases, and with increasing charge the association of peptide-type anions with sodium ions increases.

The potentiometric curves and HPLC analysis demonstrate that during the consumption of 6 equivalents of sodium the three protecting groups of oxytocein split in parallel, and not in consecutive reaction steps.

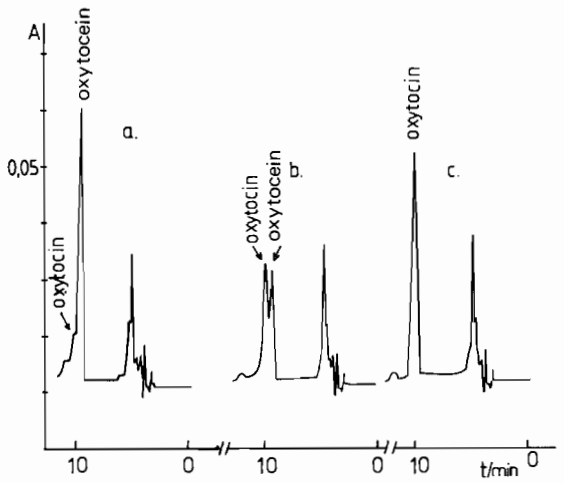


Fig. 7. Chromatograms of reduction product of protected oxytocein. Concentration of protected oxytocein in liquid ammonia: 6×10^{-3} mol dm^{-3}. Molar ratio of sodium/protected oxytocein = 9.29. (a) Analysis after 0.7 h of dissolution of reduction product in water; (b) analysis after 5.7 h; (c) analysis after 8.0 h.

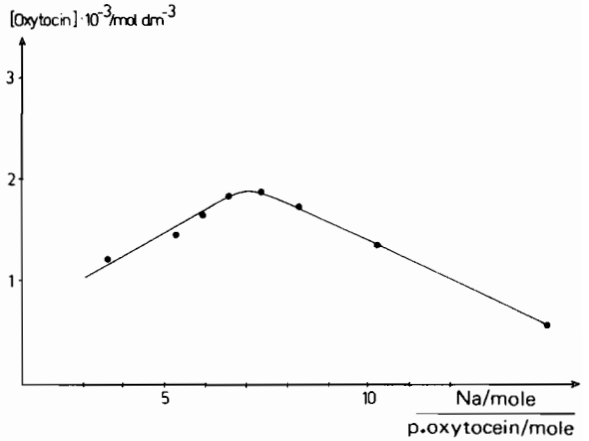


Fig. 8. Yield of oxytocin plotted against the molar ratio of sodium/protected oxytocein. Concentration of protected oxytocein in liquid ammonia: 6×10^{-3} mol dm^{-3}. Maximum possible concentration of oxytocin in water supposing 100% reduction may be 3.0×10^{-3} mol dm^{-3}.

In the reduction of protected oxytocein with sodium, primarily the sodium salt of oxytocein is formed, which is more polar than oxytocin; therefore it appears before oxytocin in the RP-HPLC conditions (Fig. 7). Oxytocin is formed from oxytocein in an oxidation reaction. For this purpose the oxygen of the air was used. In Fig. 8, the amount of oxytocin formed is plotted against the molar ratio of sodium/protected oxytocein used in the reaction. It is to be seen that the maximum yield of oxytocin is obtained at a sodium/protected oxytocein molar ratio of 6–7, in accordance with previous results [17].

Table 2 shows the material balance for the reduction of protected oxytocein. The reduction products

TABLE 2. Material Balance for the Reduction of Protected Oxytocein[a]

Concentration of protected oxytocein (mol dm^{-3})	Molar ratio of sodium:protected oxytocein	Yield of peptide after reduction[b] (%)			
		oxytocin	peptides with		total peptide
			lower mobilities than oxytocin	higher	
3×10^{-3}	6.76	83	2	12	97
	7.53	78	2	11	91
	7.65	78	7	8	93
	8.47	76	5	7	88
6×10^{-3}	7.76	67	5	13	85
12×10^{-3}	7.46	57	15	15	87

[a]The solid product of reduction was dissolved in water, pH = 6.8. [b]The percentages of side products formed in the reduction were expressed via the ratios of the areas of the corresponding peaks in the chromatograms; the concentration of oxytocin was determined on the basis of calibration with standard solutions.

were dissolved in water (pH = 6.8) and after oxidation to oxytocin, measured by HPLC. It was found that, besides oxytocin, some species with higher and lower mobility on the column than oxytocin were also formed in the reduction process. The yield of oxytocin and the amounts of other products formed depend on the reaction conditions. Further experiments are necessary to determine all the factors influencing the reaction.

Conclusions

It was shown that electroanalytical methods are suitable for the quantitative characterization of reductions of protected peptides by metallic sodium in liquid ammonia. Measuring changes in the redox potential and electric conductivity of the solution during the reduction and plotting them against the consumed volume of standard sodium solution in liquid ammonia led to titration curves reflecting the stoichiometry of the processes. Break points on the curves make the separation of consecutive reaction steps possible giving in this way information on the affinity of different functional groups to metallic sodium in the system.

The potentiometric study of the reactions of protected peptide and peptide derivatives with metallic sodium in liquid ammonia has shown that the affinity of protons to sodium, decreases in the following order: carboxyl-H > peptide bond-H ~ amide-H > primary amino-H, as mainly determined by the acidity of the protons. The potentiometric curves reflect even the effect of the surroundings on the acidity of peptide protons.

The conductometric study of the same systems reflected the mobilities of the species formed during the reactions indicating the formation of ions with different charges and association between the peptide anions and sodium cations. The measurements reflected the reaction of sodium with the solvent ammonia and the catalytic effect of ethyl thioglycolate on this reaction.

The HPLC study of the intermediate and end products of reactions of peptide and peptide derivatives with sodium has shown that the deprotonation of carboxylate and peptide bonds are reversible processes, dissolving the reaction products in water led mainly to the reestablishment of the original molecule.

The combination of the electroanalytical and HPLC measurements has shown that the three protecting groups of oxytocein split in parallel reactions in the course of reduction by sodium, the main cleavage products being oxytocein and toluene.

The complex method presented in this paper seems to be suitable for determining the optimal conditions of reductions in liquid ammonia by metallic sodium.

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