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# Reductive methylation of proteins with sodium cyanoborohydride

## Identification, suppression and possible uses of *N*-cyanomethyl by-products

Michael J. GIDLEY and Jeremy K. M. SANDERS*
*University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K.*

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Reductive methylation of protein amino groups with formaldehyde and sodium cyanoborohydride is shown to give up to 25% yield of *N*-cyanomethyl ($-CH_2CN$) product; on work up of the reaction this is hydrolysed back to starting amine, lowering the methylation yield. Addition of metal ions such as $Ni^{2+}$, which complex with free cyanide ion, improve reductive methylation yields by suppressing by-product formation. The *N*-cyanomethyl group itself, produced in good yield when cyanide ion replaces cyanoborohydride, may have some value as a *reversible* modifier of amino groups in proteins.

The reductive methylation of lysine ε-amino groups with formaldehyde and sodium cyanoborohydride (eqn. 1) is a promising method for the modification and isotopic labelling of proteins under mild conditions (Dottavio-Martin & Ravel, 1978; Jentoft & Dearborn, 1979; MacKeen *et al.*, 1979). The same chemistry has been used for the synthesis of a wide range of glycoprotein conjugates by derivatization of protein amino groups with sugar-derived aldehydes (Einarsson *et al.*, 1981; Sonenberg & Shatkin, 1977; Stowell & Lee, 1980; Vogel *et al.*, 1977; Zurawaski *et al.*, 1978):

$$R{-}NH_2 + CH_2O \rightleftharpoons R{-}\overset{\oplus}{\underset{H}{N}}{=}CH_2 \xrightarrow{NaBH_3CN} R{-}\underset{H}{N}{-}CH_3 \quad (1)$$

However, complete derivatization is never achieved, an effect which has been attributed to the competitive formation of glyconitrile, $HOCH_2CN$ (Jentoft & Dearborn, 1980); this explanation is unconvincing, as the final step of reductive methylation (eqn. 1) is irreversible and glyconitrile formation is reversible (eqn. 2). We show here that, in fact, low yields are due to *N*-cyanomethyl compounds which are formed effectively irreversibly at neutral pH by the reaction shown in eqn. (3):

$$CH_2O + CN^{\ominus} + H^+ \rightleftharpoons HOCH_2CN \quad (2)$$

$$R{-}\overset{\oplus}{\underset{H}{N}}{=}CH_2 + CN^{\ominus} \underset{\text{Very slow}}{\overset{\text{Fast}}{\rightleftharpoons}} R{-}\underset{H}{N}CH_2CN \xrightarrow{\text{Acid pH}} R{-}\overset{\oplus}{N}H_3 + CH_2O + HCN \quad (3)$$

* To whom correspondence and requests for reprints should be sent.

but which are hydrolysed to regenerate starting material on acid work-up or on prolonged dialysis at neutral pH.

### Results

In connection with proton n.m.r. studies on the anti-bacterial activity of iminium ions (Gidley *et al.*, 1981), we noticed that reductive methylation was usually accompanied by the appearance of a singlet at $\delta$ (chemical shift) ~3.7p.p.m. counting for 15–25% of the reduced material. This effect was essentially independent of the amine used, and for convenience was investigated in greatest detail for pyrrolidine. However, similar experiments are also described below for glycine, alanine and for $N^{\alpha}$-acetyl-*O*-methyl-lysine as protein models. Reaction of pyrrolidine with $^{13}C$-labelled formaldehyde and cyanoborohydride gave in the $^{13}C$ spectrum the expected *N*-methyl quartet and a triplet ($\delta$ = 44p.p.m.) coupled to protons at 3.7p.p.m. These chemical shifts were consistent with expectations for the exocyclic methylene group of *N*-cyanomethyl-pyrrolidine; the by-product was the exclusive pro-

duct from the reaction of sodium cyanide, pyrrolidine and formaldehyde, and was absent from the reaction mixture when sodium borohydride was used as reducing agent. Identification was finally secured by independent synthesis of *N*-cyanomethylpyrrolidine (Lespagnol *et al.*, 1960).

Two other by-products are generally observed in cyanoborohydride reductions. Methanol, formed by direct reduction, can account for up to 10% (but usually less than 5%) of the formaldehyde used; this problem is much more severe when borohydride itself is used. The other product, glyconitrile ($^1$H 4.6 p.p.m.; $^{13}$C 48.7 p.p.m.), is observed when formaldehyde and cyanoborohydride are present in excess, but creates no problem as its formation is reversible.

At pH 7, *N*-cyanomethylpyrrolidine is slowly hydrolysed by reversal of eqn. (3) with a $t_{\frac{1}{2}}$ of about 4 days, but the reaction is acid-catalysed and below pH 3 hydrolysis is complete in seconds. Thus reductive methylation can be carried to completion at neutral pH by trapping the iminium ion with hydride ion over a period of 10 days or so, but clearly it would be preferable to suppress by-product formation by removing cyanide ion from the reaction mixture. Jentoft & Dearborn (1980) suggested, in trying to suppress glyconitrile, that transition-metal ions could be used for this, and we have confirmed their suggestion; addition of 10 mM-nickel chloride to a pyrrolidine/formaldehyde/cyanoborohydride reaction mixture improves the ratio of the products *N*-methylpyrrolidine and *N*-cyanomethylpyrrolidine from 75:25 to 91:9. Higher concentrations of $Ni^{2+}$ would undoubtedly give further improvement, but were not studied because of line broadening in the n.m.r. assay.

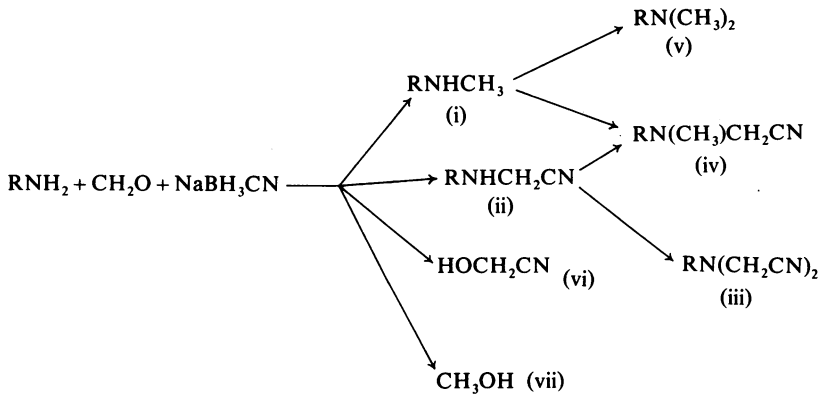
From the above results it was clear that there are seven possible products from the reaction using primary amines:

We observed six of these compounds in the reductive methylation of alanine (100 mM) with $^{13}$C-labelled formaldehyde (200 mM) and sodium cyanoborohydride (500 mM), followed by $^{13}$C n.m.r.; the chemical shifts of the resonances derived from formaldehyde are given in Table 1 together with those of the attached protons, which were correlated by a series of off-resonance decoupled spectra (Birdsall *et al.*, 1972). The only undetectable product was the bis(cyanomethyl) compound (iii). All $^{13}$C multiplicities were consistent with the assignments shown; *N*-methylalanine and bis-*N*-methylalanine were independently synthesized by straightforward methods (Borch *et al.*, 1971) for comparison purposes. Similar results were obtained with glycine under the same conditions (Table 1).

Finally, $N^{\alpha}$-acetyl-*O*-methyl-lysine was allowed to react under typical protein-reductive-methylation conditions (3.5 mM-$N^{\alpha}$-acetyl-*O*-methyl-lysine, 20 mM-formaldehyde, 25 mM-sodium cyanoborohydride, 4°C, 24 h) (Jentoft *et al.*, 1979). $^1$H n.m.r. spectroscopy showed that the ε-amino group was

Table 1. *Chemical shifts of reductive methylation products of alanine and glycine*

| | Chemical shift (p.p.m.) | | | |
|---|---|---|---|---|
| | Alanine product | | Glycine product | |
| Compound | $^{13}$C | $^1$H | $^{13}$C | $^1$H |
| (i) | 32.2 | 2.70 | 33.0 | 2.75 |
| (ii) | 35.5 | 3.65 | 36.5 | 3.65 |
| (iv) ($CH_3$ group) | 39.9 | 2.4 | 42.2 | 2.4 |
| (iv) ($CH_2CN$ group) | 42.9 | 3.75 | 45.1 | 3.75 |
| (v) | 41.7 | 2.9 | 44.4 | 2.9 |
| (vi) | 48.7 | 4.6 | 48.7 | 4.6 |
| (vii) | 49.8 | 3.4 | 49.8 | 3.4 |



completely derivatized, 84% as the *NN*-dimethyl compound and 16% as the *N*-methyl-*N*-cyanomethyl derivative ($NCH_2CN$, 3.65 p.p.m.). Addition of trichloroacetic acid (to 10%, w/v) caused rapid hydrolysis of the minor product to the *N*-methyl compound as expected. Repetition of the reaction under the same conditions with $^{13}C$-labelled formaldehyde resulted in the expected $^{13}C$ n.m.r. results: observation of methanol (49.8 p.p.m., q), glyconitrile (48.7 p.p.m., t), the *NN*-dimethyl compound (43.6 p.p.m., q) and the *N*-methyl-*N*-cyanomethyl compound (44.7 p.p.m., t; 41.9 p.p.m., q).

## Discussion

Several hitherto puzzling features of protein reductive methylation are clarified by the results described here: first, yields are never quantitative, and there always appears to be unchanged starting material present at the end of the reaction. The work described above shows that the starting material actually has reacted, but along an unproductive side-path [to our knowledge, this cyanomethylation has been reported only once before in a reductive reaction, and that was under very different reaction conditions (Lown & Itoh, 1975)]. Second, the maximum reported derivatization yield of 1.85 mol of formaldehyde per free amino group (Jentoft & Dearborn, 1979) is in excellent agreement with our experiments on protected lysine. Third, an unidentified $^{13}C$ n.m.r. signal at 44.5 p.p.m. has been reported (Jentoft *et al.*, 1979) after short periods of dialysis after reductive methylation; the signal can now be identified as arising from the *N*-methyl-*N*-cyanomethyl compound. This product is not normally observed, since either long periods of dialysis at neutral pH or brief treatment with acid will hydrolyse it to starting material. Fourth, and finally the addition of metal ions to increase the yield is effective not because it prevents the formation of the labile and inocuous glyconitrile, but because it suppresses the more damaging formation of *N*-cyanomethyl derivatives. Recrystallization of sodium cyanoborohydride to remove adventitious cyanide ion is also beneficial for the same reason. An alternative possibility might be to use aminoborane reducing agents (Geoghegan *et al.*, 1981).

The *N*-cyanomethyl group itself may have some interest and application as a *reversible* lysine modifier. It is formed in good yield by reaction with formaldehyde and cyanide ion in the absence of hydride reducing agent, but is easily removed under a range of mild conditions: several days' dialysis at neutral pH or in seconds below pH 3. It would therefore be used to protect the lysine amino group (or, more subtly, the most reactive amino group), allowing some other modification or process to be carried out elsewhere in the protein. In addition, the *N*-cyanomethyl group has the potential for further modification, e.g. by mild hydrolysis to carboxylate ($NCH_2CO_2^-$).

## Materials and methods

Amino acids and sodium cyanoborohydride were obtained from Sigma Chemical Company and Aldrich respectively and were used without purification. $^{13}C$-labelled paraformaldehyde was a gift from Geistlich and Sons Ltd., Wolhusen, Switzerland.

Proton n.m.r. spectra were recorded in the Fourier-transform mode at 80 or 100 MHz on Varian CFT20 and XL100 spectrometers respectively; 25.2 MHz $^{13}C$ spectra were obtained on the latter instrument. All spectra were obtained on $^2H_2O$ solutions with a $^2H$ lock; chemical shifts ($\delta$) are in p.p.m. downfield from tetramethylsilane, and were calculated by using internal standards of sodium 3-(trimethylsilyl)propionate ($^1H$, 0 p.p.m.) or dioxan ($^{13}C$, 67.4 p.p.m.). Accuracy of integrals was ensured by using a pulse repetition time of greater than five times the longest $T_1$ in the system under study. All spectra were measured at ambient probe temperature (30–35°C).

Commercial aqueous solutions of formaldehyde (formalin) contain 10% (v/v) methanol as stabilizer and were unsuitable. Concentrated aqueous formaldehyde and [$^{13}C$]formaldehyde solutions were prepared by heating paraformaldehyde and water (or $^2H_2O$) at 110°C in a sealed tube for about 16 h. Dilute (< 5%, w/v) solutions were obtained by autoclaving an aqueous suspension of paraformaldehyde at 115°C and 101.3 kPa (1 atm) for 30 min. Yields were more than 95% as judged by the sodium sulphite assay (Walker, 1964). *N*-Cyanomethylpyrrolidine was prepared by the method of Lespagnol *et al.* (1960) in 73% yield (b.p. 116–118°C).

### *N-Methylglycine*

Its synthesis was based on the method of Borch *et al.* (1971). Glyoxylic acid hydrate (0.92 g, 10 mol) in methanol solution (100 ml) was adjusted to pH 6 with KOH; methylamine hydrochloride (3.4 g, 50 mmol) was added, followed by sodium cyanoborohydride (1 g, 20 mol). The mixture was stirred for 72 h at room temperature, concentrated HCl (50 ml) was added and stirring was continued for a further 1 h. Solvent was removed under reduced pressure, and the residue was taken up in water (25 ml) and applied to a Dowex 50 ($H^+$ form) column. The column was washed with water (1 litre) and eluted with 1 M-$NH_3$ (1 litre). Evaporation of solvent gave essentially pure product. The yield after recrystallization from ethanol/acetone was 35%; m.p. 211°C (decomp.); literature value 212°C (Weast, 1967).

*NN-Dimethylglycine*

This was synthesized as above, but dimethylamine hydrochloride was used. [Yield 38%, m.p. 180°C; literature value 178–182°C (Bowman & Stroud, 1950).]

*N-Methylalanine*

This was synthesized similarly in 52% yield from pyruvic acid and methylamine hydrochloride [m.p. 286°C (decomp.); literature value 292°C (decomp.) (Weast, 1967)].

*NN-Dimethylalanine*

This was synthesized as described above from pyruvic acid and dimethylamine hydrochloride in 57% yield [m.p. 189°C; literature value 188°C (Bowman & Stroud, 1950)].

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