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N-demethylation of cyamemazine via non-classical Polonovski reaction and its conjugation to bovine serum albumin

Gurmit Singh [a,*], Terry B. Koerner [a], Samuel Benrejeb Godefroy [b], Claude Armand [c]

[a] Food Research Division, Bureau of Chemical Safety, Health Canada, AL2203D, Ottawa, ON, Canada K1A 0L2
[b] Food Directorate-HPFB, Health Canada, Ottawa, ON, Canada K1A 0L2
[c] Substipharm Développement, 8 Rue Bellini, 75116 Paris, France

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ABSTRACT

A modified Polonovski reaction has been employed to obtain the N-demethylated metabolite of the neuroleptic drug cyamemazine. The synthesis involves *N*-oxide formation, isolation of the corresponding *N*-oxide, and a $FeSO_4 \cdot 7H_2O$ mediated Polonovski reaction to afford the desired monodesmethyl cyamemazine. In a subsequent step the hapten *N*-demethylcyamemazine-hemiglutarate was synthesized and its conjugated to bovine serum albumin (BSA).

The phenothiazine derivative cyamemazine was originally developed as an antipsychotic agent targeting central dopamine receptors, but has since shown anxiolytic activity and efficacy in relieving the symptoms of alcohol and benzodiazepine withdrawal.[1,2] Cyamemazine is thought to be metabolized in the liver into the two main metabolites monodesmethyl cyamemazine (**3**) and cyamemazine sulfoxide (**5**, Fig. 1). A recent study using recombinant human liver microsomal enzymes of cytochrome P450 has confirmed these metabolic products and suggested the minor metabolites *N*,*N*-demethylated and N-demethylated mono-oxidized products.[3] It is interesting to note that the mono-demethylated metabolite has a high affinity for the same receptors as cyamemazine and is thought to contribute to the prolonged therapeutic action.[4] Due to the numerous medical conditions for which cyamemazine has been approved it has become widely prescribed and thus has a greater potential to be combined with other medications.[5] It has been suggested that the multiple P450 enzymes involved in the clearance of cyamemazine would limit the likelihood of other therapeutic agents inhibiting the detoxification, but little work has been done on the interaction of cyamemazine metabolites with other therapeutic agents.[3]

The metabolic products of therapeutic agents are typically not commercially available, which was the case for cyamemazine so a synthetic route to the mono-demethylated metabolite of cyamemazine needed to be developed for pharmacological studies.

To our knowledge there is no information in the literature regarding the direct synthetic conversion of cyamemazine to its mono-demethylated metabolite. The reaction involves the conversion of the amine to the corresponding *N*-oxide using a suitable peroxy acid followed by activation to induce the elimination, formation of an iminium ion intermediate and finally the demethylated product. The main classes of agents that have been used to activate the *N*-oxide are acylating agents (acid anhydrides and chlorides), iron salts, and sulphur dioxide. This non-classical Polonovski reaction using iron salts has been used for the N-demethylation of a range of compounds, but it has been most successful when the tertiary nitrogen is part of a ring system.[6] The N-demethylation of galanthamine to norgalanthamine,[7] and the N-demethylation of opiate alkaloids[8] are examples. In case where the tertiary nitrogen is not part of a ring system the results can vary depending on reaction conditions and, if the nitrogen is in a favourable position, ring closure can occur.[9,10]

Literature reports have suggested a biological route from cyamemazine to monodesmethyl cyamemazine by the cytochrome P450 enzymes,[3] but an adequate chemical model has yet to be demonstrated for this demethylation reaction. We initially attempted the conversion of cyamemazine into monodesmethyl cyamemazine by using a ruthenium-catalyzed reaction[11] and a reaction using pyridinium chlorochromate,[12] but these reactions were unsuccessful. In both of these examples the demethylation occurred when tertiary nitrogen was adjacent to aromatic ring.

The desired transformation was accomplished by a non-classical Polonovski reaction using iron salts. This approach involves the conversion of the amine to the corresponding *N*-oxide using

* Corresponding author. Tel.: +1 613 957 0977; fax: +1 613 941 4775.
E-mail address: gurmit.singh@hc-sc.gc.ca (G. Singh).

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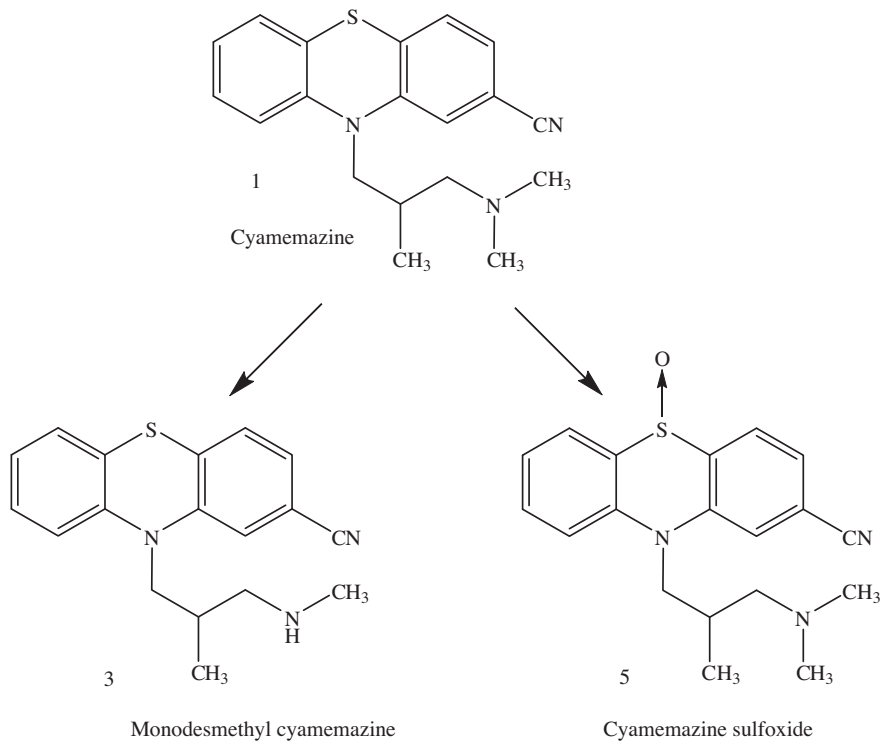
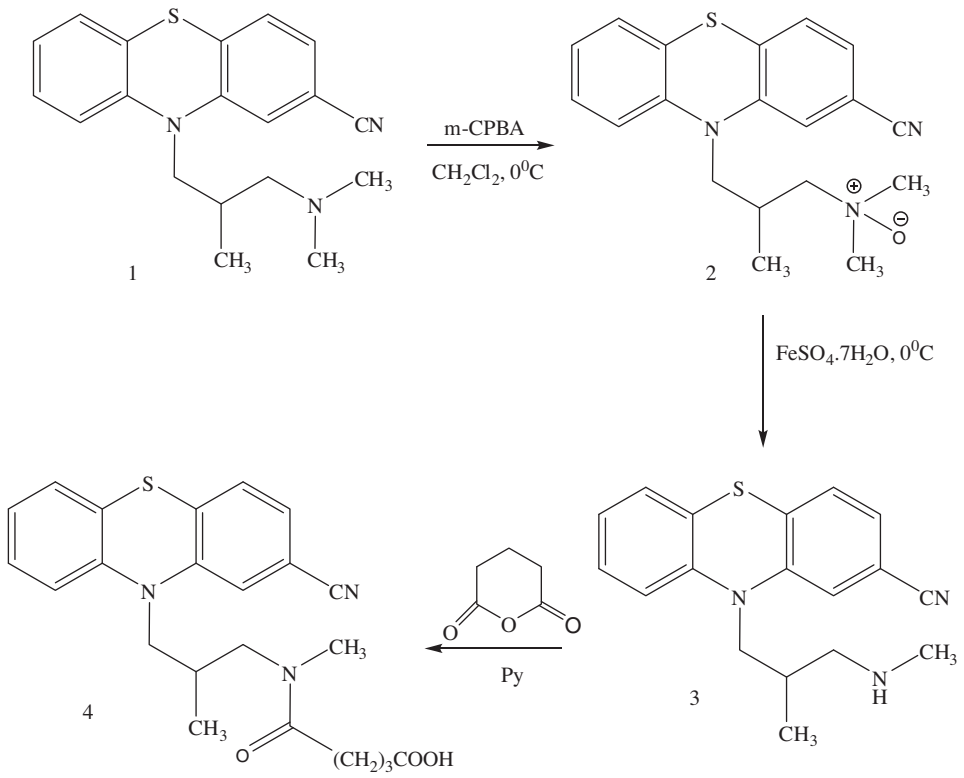
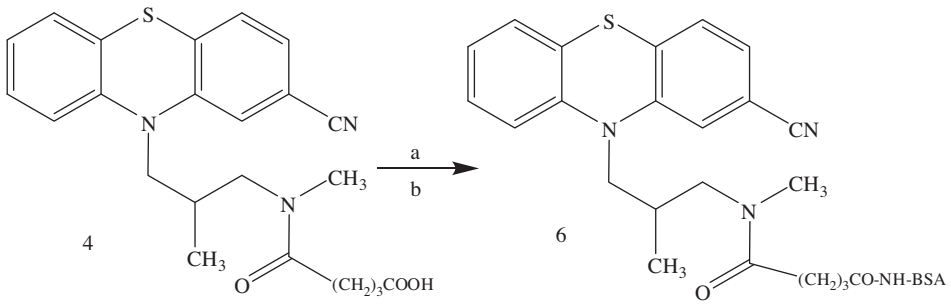


Figure 1. Structures of cyamemazine and its two main metabolites.

m-chloroperbenzoic acid. The *N*-oxide then reacts with an iron(II) sulphate heptahydrate, which induces elimination and ultimately affords an iminium ion intermediate that reacts further to yield the N-demethylated product. In the first step (Scheme 1), cyamemazine (**1**) was transformed into cyamemazine *N*-oxide (**2**)[13] by oxidation with *m*-chloroperbenzoic acid in dichloromethane at 0 °C. Subsequent treatment of cyamemazine *N*-oxide with hydrated ferrous sulphate in methanol at 0 °C provided monodesmethyl cya-



Scheme 1. Synthetic protocol of the monodesmethyl cyamemazine and hapten.



Scheme 2. Synthesis the immunogen: (a) ethylchloroformate, triethylamine, (b) BSA.

memazine (**3**)[14] with an overall yield of 70%. The mechanistic aspects of this reaction have been described in the literature, but it is thought the reaction involves two successive one-electron steps to form an iminium ion, which is hydrolysed to the secondary amine.[8] The parent amine is the major side product when the tertiary nitrogen is connected to a ring system, but aldehyde formation is common in aliphatic systems.[6] The N-demethylation step was carried out using methanol as the solvent instead of using acidic solution to avoid aldehyde formation.[15]

The potential for ring closure, which has been observed[9,10] for similar reactions during the N-demethylation of cyamemazine, is reduced because it would require the formation of an eight membered ring system rather than the more stable six member system. The final step in the synthesis was the formation of the cyamemazine-hemiglutarate (**4**),[16] which was conjugated with BSA (Scheme2) will be used as immunogen(**6**)[17] in attempt to generate either polyclonal or monoclonal antibodies.

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13. Synthesis of cyamemazine *N*-oxide (**2**): The first step of the reaction involved the addition of 0.5 mL of a NaOH solution (4 N) to a suspension of cyamemazine tartrate (0.646 g, 2 mmol) in dichloromethane (15 mL). The mixture was stirred until the solid had dissolved, cooled to 0 °C and treated with *m*-chloroperbenzoic acid (0.344 g, 2 mmol) in several portions. This mixture was stirred for four hours at a constant temperature (0 °C) at which time the reaction mixture was allowed to warm to room temperature and the solvent evaporated in a vacuum yielding a crude yellow product. Workup was accomplished by dissolving the material into distilled water (20 mL), basified (pH 10) with NaOH (1 N) and extracting with CH_2Cl_2 (3 × 20mL). The organic extracts were combined, dried over $MgSO_4$, filtered and the solvent removed to concentrate the sample before purification by column chromatography. The crude material was loaded onto a silica gel column, eluted with an ethyl acetate/methanol mixture (3:2) to yield the title compound as yellow oil after solvent removal. Yield 70%; ^{1}H NMR (400 MHz, CD_3OD): δ 7.39–7.30 (m, 4H), 7.22–7.15 (m, 2H), 7.07–7.03 (m, 1H), 4.05 (dd, J = 8.0, 13.9 Hz, 1H), 3.82 (dd, J = 8.0, 13.9 Hz, 1H), 3.38 (dd, J = 6.1, 12.9 Hz, 1H), 3.24 (dd, J = 6.1, 12.9, 1H), 3.09 (s, 3H), 3.07 (s, 3H), 2.62–2.59 (m, 1H), 1.26 (d, J = 6.7 Hz, 3H). ^{13}C NMR (CD_3OD): 146.76, 144.30, 133.36 128.37, 128.20, 127.75, 126.61, 124.86, 123.97, 119.07, 118.66, 117.58, 111.37, 74.75, 58.19, 57.84, 51.81, 27.47, 17.78. EI-MS $[M+H]^+$; calcd. For $C_{19}H_{21}N_3OS$, 339.14; found 340.21.
14. Synthesis of monodesmethyl cyamemazine (**3**): Cyamemazine-*N*-oxide (0.158 g, 0.46 mmol) was dissolved in methanol (8 mL) and cooled in an ice-water bath to which a solution of $FeSO_4{\cdot}7H_2O$ (0.258 g, 0.93 mmol) in methanol (1 mL) was added. This mixture was stirred at 0 °C for 2 h and the solvent was removed to yield a reddish solid, which was dissolved in an EDTA solution (0.1 M at pH 10 using NH_3 solution) and extracted with $CHCl_3$ (3 × 20 mL). The organic layers were dried over $MgSO_4$, filtered and the solvent removed to yield a mixture of monodescyamemazine and cyamemazine. Monodesmethyl cyamemazine was purified by preparative liquid chromatography plate (PLC) (Whatmen RFC18 F plate) methanol:acetone (1:1). Yield 70%; ^{1}H NMR (400 MHz, CD_3OD): δ 7.30–7.22 (m, 4H), 7.18–7.14 (m, 1H), 7.07–6.99 (m, 2H), 3.92 (dd, J = 7.1, 13.8 Hz, 1H), 3.75 (dd, J = 7.1, 13.8 Hz, 1H), 3.32 (br, 1H), 2.68 (dd, J = 6.1, 11.8 Hz, 1H), 2.44 (dd, J = 6.1, 11.8 Hz, 1H) 2.31 (s, 3H), 2.29–2.21 (m, 1H), 1.01 (d, J = 6.7 Hz, 3H). ^{13}C NMR (CD_3OD), 146.45, 144.42, 133.02, 127.78, 127.68, 127.22, 125.90, 124.33, 123.29, 118.50, 118.37, 116.58, 110.65, 55.84, 51.79, 35.08, 30.19, 15.02. HRMS (EI) calcd. For $C_{18}H_{19}N_3S$, 309.1300; found 309.1308.
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16. Synthesis of monodesmethyl cyamemazine-hemiglutarate (**4**): Monodesmethylcyamemazine (15 mg; 0.05 mmol), was reacted with 6 mg (0.05 mmol) of glutaric anhydride in the presence of 2 mL of pyridine. The reaction was stirred overnight at room temperature. The extent of the reaction was ascertained using silica gel thin layer chromatography (TLC) developed by ethyl acetate: hexane (3:2). After the reaction was complete, the pyridine was evaporated under a stream of nitrogen and the crude product was purified using PLC (Yield 75%). ^{1}H NMR(400 MHz, CD_3OD): δ 7.31–7.27 (m, 4H), 7.17–7.16 (m, 1H), 7.04–6.99 (m, 2H), 3.92 (dd, J = 7.1, 13.8 Hz, 1H), 3.93 (dd, J = 6.7, 13.8 Hz, 1H), 3.71 (dd, J = 6.7, 13.8, 1H), 3.57 (dd, J = 6.7, 13.8, 1H), 3.25 (dd, J = 6.7, 13.8, 1H) 2.98 (s, 3H), 2.43–2.31 (m, 5H), 1.77–1.75 (m, 2H), 1.01 (d, J = 6.7 Hz, 3H). ^{13}C NMR (CD_3OD), 175.56, 173.87, 146.38, 144.10, 132.74, 127.83, 127.78, 127.25, 125.87, 124.21, 123.28, 118.36, 118.34, 116.44, 110.64, 53.64, 51.87, 35.12, 34.09, 32.08, 31.47, 19.93, 14.95. HRMS (EI) calcd. For $C_{23}H_{25}N_3O_3S$, 423.1617; found 423.1625.
17. Conjugates of monodesmethyl-cyamemazine-hemiglutrate with BSA. Monodesmethylcyamemazine-hemiglutrate(15 mg) was dissolved in 2 mL of DMF and 1,4 dioxane (1:1) and triethylamine (10 μL) was added to the reaction mixture and stirred on ice for 10 min. Ethyl chloroformate (10 μl) was added and the reaction mixture was allowed to warm to room temperature and stirred for one hour. This mixture was added to an ice-cold BSA solution (35 mg BSA, dissolved in 3 mL of 0.1 M sodium borate) and stirred overnight at room temperature. The final solution was dialyzed using 12–14 kDa tubing against several changes of buffer (PBS) at 4 °C for 3 days then lyophilized. The molar ratio of cyamemazine hapten per carrier protein was estimated by measuring the free amino groups on the lysine side chains after reaction with TNBS reagent. The differential absorption of the native and the conjugated proteins at 420 nm allowed the determination of an average conjugation ratio.