SIMPLE PROCEDURES FOR PREPARING PUTATIVE TOXIC METABOLITES OF PYRROLIZIDINE ALKALOIDS

A. R. MATTOCKS, REBEKAH JUKES and JANET BROWN

Toxicology Unit, M.R.C. Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K.

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A. R. MATTOCKS, R. JUKES and J. BROWN. Simple procedures for preparing putative toxic metabolites of pyrrolizidine alkaloids. *Toxicon* **27**, 561-567, 1989.—New procedures are described for converting unsaturated pyrrolizidine alkaloids to chemically reactive pyrrolic esters (dehydro-alkaloids), which are probable primary toxic metabolites formed from these alkaloids in vivo. Preparations of dehydro-secines, including dehydroretroeneine (DHR) are also described. Dehydrocrotonine, and four new dehydro-alkaloids, are described for the first time. The methods give superior yields to earlier procedures, do not require a high degree of chemical expertise, and are particularly suitable for making small amounts of compounds for toxicological experiments.

INTRODUCTION

Unsaturated pyrrolizidine alkaloids (PAs) are hepatotoxins and carcinogens found in various plant species throughout the world (Smith and Culvenor, 1981; Mattocks, 1986). They represent a serious health risk not only to grazing livestock, but sometimes also to human populations which may be exposed to them either through contamination of foods or in herbal teas or medicines (Huxtable, 1980; Schoental, 1982; Mattocks, 1986). Pyrrolic metabolites (dehydro-alkaloids) formed by metabolic dehydrogenation of alkaloids such as monocrotaline (Fig. 1a) in the liver, are held to be responsible for toxic actions not only in the liver but also in the lungs and sometimes other tissues (Mattocks, 1972; Huxtable, 1980). Since the first demonstration that pyrrolic esters prepared chemically from PAs are considerably more active hepato- and pneumotoxins than the parent alkaloids (Butler et al., 1970), these pyrroles have been increasingly used in experimental toxicology. For instance, dehydromonocrotaline (Fig. 1b) (monocrotaline pyrrole) is used to produce pulmonary injury and hypertension in animals as a model of human cardiopulmonary disease (Cheesney et al., 1974; Lafranconi and Huxtable, 1981); the somewhat more stable pyrrole dehydro-anacrotine (Fig. 1j) has similar activity (Mattocks and Driver, 1987).

Pyrrolic ester derivatives (dehydro-PAs) are reactive and unstable; they polymerize in the presence of moisture and acids and cannot be stored at room temperature or in solution. They are best prepared from the parent alkaloids in small quantities, shortly before use. Dehydro-secines are pyrrolic alcohols derived from the secine bases (e.g. Fig. 1d,e) which constitute the amino alcohol moiety of PAs. They are more water soluble and somewhat...
less unstable than the pyrolic esters. We have found that difficulties are often encountered by workers with limited chemical experience attempting to make and purify dehydro-PAAs and -necines. Thus, there is a need for simpler procedures for preparing these compounds quickly and easily, with good yields and purity. This paper describes several such procedures, which are easier to carry out than older methods and give superior yields.

Methods for preparing dehydro-PAAs involve either the dehydrogenation of the basic alkaloids with various oxidizing agents, or dehydration of the alkaloid N-oxides (Mattocks, 1968a, 1969, 1981; Culvenor et al., 1970a, b). Thus, the most convenient practical method for making dehydromonocrotaline (Fig. 1b) has been to treat monocrotaline N-oxide with ferric sulphate in methanol solution (Mattocks, 1969). The pyridine alcohol dehydroretrocotecine (DHR) (Fig. 1f) can be similarly prepared from the N-oxide of retrocotecine (Fig. 1d). Basic PAAs and necines can be converted to pyroles using oxidizing agents such as manganese dioxide or potassium permanganate, with 'stable' free radicals such as diphenylpicrylhydrazyl or potassium nitrosodisulphonate (Fremy's salt), or with reactive quinones. Among the latter, p-chloranil (tetrachloro-1,4-benzoquinone) has been used to prepare DHR (Fig. 1f) and dehydroheliotridine (Culvenor et al., 1970a), but its solubility is low, and the products are difficult to purify. PAAs are readily dehydrogenated by dichlorodicyanobenzoquinone (DDQ) (Mattocks, 1969), and extension of this reaction to

Alkaloids and related substances

The PAs used in this study were obtained from the following sources:

- Monocrotaline (MCA)
- 2,3-Dihydro-5,6-dimethoxy-4-methylenenitroso-1,2-benzoquinone
- Thin layer chromatography

Thin layer chromatography of the following was performed using (a) acetate-nitrite, followed by Ehrlich reagent only. The spots were detected under u.v. light. Mass spectroscopy

Preparation of dehydro-PAAs

1. Using p-chloranil (10% solution) (1 ml) to separate, immediate dehydro-alkaloid

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Anacrotine</th>
<th>Fulvine</th>
<th>Heliotroline</th>
<th>Integerrimine</th>
<th>Lasiocarpine</th>
<th>Monocrotaline</th>
<th>Retrocotecine</th>
<th>Scopoeclline</th>
<th>Supinine</th>
</tr>
</thead>
</table>
Preparations of Pyrrolizidine Alkaloids

A preparative scale is described here. o-Chloranil (tetrachloro-1,2-benzoquinone) was introduced by Molynieux and Rottman (1980) for detecting PAs on TLC, and more recently it has been used in a test for PAs in plant extracts (Mattocks and Jukes, 1987). We have found that o-chloranil is very useful for the preparative dehydrogenation of PAs, because it is highly soluble in organic solvents, reacts rapidly, and breaks down to products which are easy to remove. The related reagent o-bromanil gives similar results and even purer products, and is especially useful for making dehydro-neomes.

MATERIALS AND METHODS

Alkaloids and reagents

The PAs used, and their plant sources, were as listed in Table 1. Retronecine (Fig. 1d) was prepared by hydrolysis of monocrotaline (Adams and Rogers, 1979), similarly crotanecine (Fig. 1c), by hydrolysis of macrotin (Mattocks, 1968) and supinine from supinine.

2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), tetrachloro-1,2-benzoquinone (o-chloranil) and tetrabromo-1,2-benzoquinone (o-bromanil) were from Aldrich Chemical Co.

TLC (layer chromatography / TLCs), TLCs were run on silica-coated aluminium sheet (Merck 5554) using either of the following solvents: (1) light petroleum (b.p. 60-80°C)-acetone, 1:1; (2) butanone-acetone, 2:1; (3) ethyl acetate-acetone-ethyl alcohol aqeous ammonia, 5:3:1:1. Alkaloids were visualized by spraying with o-chloranil followed by Ehrlich reagent (Molynieux and Rottman, 1980): dehydro-alkaloids were spayed with Ehrlich reagent only. Rf values are shown in Table 2.

Mass spectra. Positive or negative ion FAB spectra were run in a glycerol matrix, on a VG 70 SEQ mass spectrometer.

Preparation of dehydro-pyrrolizidine alkaloids

1. Using o-chloranil. To the alkaloid (20 mg) dissolved in chloroform (5 ml) was added at room temp a solution of o-chloranil (25 mg) in CHCl3 (1 ml). After 1.5-2 min the mixture was shaken vigorously with a cooled (5-10°C) solution (1 ml) containing KOH (70%) and sodium borohydride (2%) for 10-15 sec. The organic phase was separated, immediately dried (Na2SO4) and concentrated under reduced pressure to give the practically pure dehydro-alkaloid (75-100%). If the CHCl3 solution was still coloured after the KOH wash, it was either washed a second time, or decolourised with charcoal after being dried.

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Source plant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anacrotine</td>
<td>Crotalaria incanus (South Africa)</td>
<td>Mattocks (1968)</td>
</tr>
<tr>
<td>Fulvinine</td>
<td>Crotalaria salvi (Jamaica)</td>
<td>Schoental (1963)</td>
</tr>
<tr>
<td>Heliotrine</td>
<td>Heliotropium supinum (India)</td>
<td>Pandy et al. (1983)</td>
</tr>
<tr>
<td>Intermineine</td>
<td>Seneio brasiliensis (Brazil)</td>
<td>Schmeda Hirschmann et al. (1987)</td>
</tr>
<tr>
<td>Lasiocarpine</td>
<td>Heliotropium supinum (India)</td>
<td>Pandy et al. (1983)</td>
</tr>
<tr>
<td>Monocrotaline</td>
<td>Crotalaria spectabilis (seed)</td>
<td>Constantinou et al. (1967)</td>
</tr>
<tr>
<td>Retinosine</td>
<td>Senecio inuricis (South Africa)</td>
<td>Koekemoer and Warren (1951)</td>
</tr>
<tr>
<td>Senecionine</td>
<td>(Semisynthetic, from retinosine)</td>
<td>Mattocks (1983)</td>
</tr>
<tr>
<td>Riddelline</td>
<td>Senecio ridellii (United States)</td>
<td>Molynieux and Johnson (1984)</td>
</tr>
<tr>
<td>Supinine</td>
<td>Trichosperm zeylanicum (seed)</td>
<td>O'Kelly and Sargeant (1951)</td>
</tr>
</tbody>
</table>
Compounds prepared as above included: dehydrocorynantheine (Fig. 1), yield 93%; \textit{m}z (+ FAB) 350 (MH)+; 269, 226, 191, 134 (base), 118, 106; dehydrokuninoline (Fig. 1c), yield 96%; \textit{m}z (+ FAB) 328 (MH)+; 210, 134, 120, 106, dehydro-\textit{tetrabenzil}one (1k), yield 100%; \textit{m}z (+ FAB) 344 (MH)+; 210, 164, 134, 136, 120.

The reaction was easily scaled up. Thus, monomethacrine (100 mg) in CHCl3 (45 ml) with o-chloranil (125 mg) in CHCl3 (15 ml) yielded crystalline dehydrocorynantheine (Fig. 1b) (98 mg, 100%)

2. Using o-bromanil, o-bromanil could replace o-chloranil in the above procedure. Thus, riddelline (20 mg) and o-bromanil (30 mg), each in CHCl3 (3 ml) were mixed, kept 1.5 min, diluted with CHCl3 (8 ml), then worked up as above to give crystalline dehydrodehydorindoline (Fig. 1d) (18.5 mg 93%), \textit{m}z (+ FAB) 348 (MH)+; 210, 134, 120 (base), 162.

3. Using DDQ, the solution of the alkaloid base (20 mg) in chloroform (2 ml) at room temp was added a solution of DDQ (25 mg) in tetrahydrofuran (0.5 ml) and chloroform (2 ml). After 2 min the mixture was passed through a column of alumina (neutral grade 1) (1 g) and eluted with further CHCl3, until a drop of the eluate ceased to react with Ehrlich reagent. Alternatively, it was shaken repeatedly with small volumes (0.5-2 ml) of a cooled (5-10°C) solution containing KOH and NaBH4 (as above), until the organic phase was colourless (five to eight washes needed). The chloroform solution was quickly dried (Na2SO4) and evaporated under reduced pressure leaving the dehydro-alkaloid (60-90%)

Purification of dehydro-pyrrolizidine alkaloids

The above procedure provided practically pure dehydro-alkaloids. Products made using DDQ or o-chloranil (but not o-bromanil) sometimes contained traces of the basic parent alkaloid (which would help to stabilize the acidic product). Those which were crystalline could be recrystallized from mixtures of benzene and light petroleum, with the exclusion of moisture.

Preparations of dehydro-\textit{neocoryne}, Dehydrocorynantheine (DH)

1. Solutions of \textit{retrocucar}nine base (100 mg) in CHCl3 (30 ml), were cooled to 0-5°C and mixed. After 10 sec a cold (0-5°C) solution of KOH + NaBH4 (as above) was added and the mixture was shaken vigorously for 0.5-1 min. The pale violet organic phase was separated, dried (Na2SO4), and filtered, washed with a little activated charcoal, filtered, and evaporated at 45-50°C under reduced pressure to give DH (Fig. 1f) (63 mg, 64%) which crystallized after rubbing with ether, m.p. 84-86°C. It was purified by dissolving it in a minimum of warm benzene, then diluting with a large volume of ether. If slightly cloudy, the mixture was further diluted with ether, charcoal filtered, and dried. The liquor was concentrated to a small volume, and light petroleum (b.p. 60-80°C) was added. Seeding with DH gave colourless prisms, m.p. 89.5-90°C [L]31 91' 9°C. CHEVIGNON et al., 1932, 8688. MATTOCKS, 1981. Re-extraction of the aqueous wash liquid (CHCl3, x 1) yielded further product (17 mg), but this contained up to 3.5 mg (17%) of unreacted \textit{retrocucar}nine as shown by NMR analysis.

2. Using o-bromanil. Anion exchange resin (Bio-Rad AG508, 200-400 mesh in OH form (g) was washed with water, then with methanol, and the excess solvent was expelled by air pressure prior to use. Solutions of \textit{retrocucar}nine base (100 mg) in CHCl3 (35 ml) and o-bromanil (300 mg, 100% of theoretical) in CHCl3 (15 ml) were cooled to 0-5°C, then mixed. After 15 sec the resin was added and the mixture was shaken vigorously in a stopped flask un

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
\textbf{Dehydro (DH) compound} & \textbf{Physical form and m.p.} & \textbf{TLC Rf in (solvent)} & \textbf{Rf of parent alkaloid in solvent 3} \\
\hline
DH-corynantheine (I) & Cryst. (dec.) & 0.49 & 0.64 & 0.69 & 0.38 \\
DH-furulin (C) & Cryst. (dec.) & 0.55 & 0.67 & 0.83 & 0.38 \\
DH-\textit{deido} (C) & Gum & 0.50 & 0.60 & 0.58 & 0.49 \\
DH-\textit{tetrabenzil}one (k) & Cryst. (111-112°C) & 0.62 & 0.70 & 0.78 & 0.35 \\
DH-\textit{la} (C) & Gum & 0.56 & 0.68 & 0.74 & 0.48 \\
DH-monocorynantheine (b) & Cryst. (dec.) & 0.48 & 0.60 & 0.66 & 0.29 \\
DH-\textit{retrocucar}nine (G) & Cryst. (dec.) & 0.58 & 0.60 & 0.60 & 0.36 \\
DH-\textit{neocoryne} (d) & Cryst. (121°C) & 0.63 & 0.69 & 0.75 & 0.37 \\
DH-\textit{dihydro} (f) & Cryst. (90°C) & 0.23 & 0.40 & 0.47 & 0.20 \\
DH-\textit{dihydro} (g) & Cryst. (78.5°C) & — & — & 0.27 & 0.10 \\
DH-\textit{dihydro} (i) & Oil & — & 0.38 & 0.65 & 0.32 \\
\hline
\end{tabular}
\caption{Physical and TLC characteristics of dehydro-pyrrolizidine alkaloids and dehydro-neocoryne.}
\end{table}

\*Crystalline dehydro-alkaloids often decomposed near 100°C without melting.

\#New compound.

Compound 1 were new, the publication (MATTOCKS, 1981) was isolated in excess of reagent color to 2 min even sensitive to moisture, washed with a mixture of ether, which catalyzes reaction products. Re-extraction with wash liquor did not give a product. NMR. The loss of the material was probably due to greater

Attempts to prepare dehydrospinosamide yield to DH.

Dehydrospinosamide procedure. It was hoped to limit the reaction time in preparative scale, but a number of products could be recrystallized. Nevertheless, some of the products were not obtained yields of DH were obtained in the same way.
Preparations of Pyrrolizidine Metabolites

Stopped flask until the liquid phase was colourless (1–2 min), then filtered (pump). The resin bed was washed with several lots of CHCl₃. The combined filtrates were concentrated at 40°C under reduced pressure, yielding crystalline DHR (99 mg, 100%). The product contained no unchanged retronecine as shown by TLC and NMR. Recrystallization from benzene-light petroleum (charcoal) gave prisms, m.p. 89–90°C.

1. Dehydrotropane. Cretonine (46 mg) was dissolved in warm methanol (1 ml), dilute with CHCl₃ (20 ml) and the solution was cooled to 0–5°C and mixed with a solution of o-bromamal (120 mg; 120% of theoretical) in CHCl₃ (5 ml), similarly cooled. After 15 sec the solution was shaken vigorously with two successive lots of water (2 ml, 1 ml) which were separated, combined and then washed once with ether (5 ml). The aqueous solution was freeze dried to give a gum, which crystallized. Residual water was removed by adding benzene and evaporating this at 40°C under reduced pressure. The product (30 mg, 75%), m.p. 70°C, was recrystallized three times from acetone-light petroleum (b.p. 60–80°C) to give dehydrotropane (Fig. 1) as colourless blades, m.p. 78.5°C, m/z (–FAB) 169 (M–H) (100%), m/z (+FAB) 169 (M) + (15%), 152 (base), 138.

2. Dehydrosupinine. Solutions of supinine (35 mg) in CHCl₃ (20 ml) and o-bromamal (110 mg) in CHCl₃ (5 ml) were mixed together at 0–5°C. After 20 sec the pale green solution was shaken with KOH + NaBH₄ solution (as above) (2 ml) for 1 min. The CHCl₃ phase was separated, the aqueous phase was washed twice with CHCl₃ (10 ml), and the combined CHCl₃ was dried (K₂CO₃) and concentrated under reduced pressure to give dehydrosupinine (Fig. 1) as a colourless oil (27 mg, 77%). TLC showed a single spot and the NMR spectrum was as previously reported (CULVENOR et al., 1970a).

RESULTS AND DISCUSSION

Compounds prepared using the above methods are listed in Table 2. Five of these (e. g. j–l) were new, including dehydro-anacrotine (j) which has been mentioned in an earlier publication (MATTOCKS and DRIVER, 1987); the remainder have been described previously (MATTOCKS, 1969; CULVENOR et al., 1970a). The products were practically pure, and easily isolated in excellent yields. Reactions were very fast, as indicated by a reduction in the reagent colour within a few seconds. However, an excess of quinone and reaction times up to 2 min ensured that a minimum of alkaloid remained unreacted. Dehydro-PAs are sensitive to moisture, but they are quite lipophilic, so that a solution in chloroform could be washed with a minimal volume of aqueous solution provided that the contact time was kept short, the temperature was low, and a high level of alkali was used to suppress H⁺ ions which catalyse hydrolysis and polymerization. Several washes were needed to remove DDQ reaction products, but one wash was usually sufficient after o-chloramal or o-bromamal reactions. Removal of unreacted reagent was facilitated by including borohydride in the wash liquor. There was little to choose between o-chloramal and o-bromamal, but the latter gave products completely free from traces of parent alkaloid, as indicated by TLC and NMR. The lowest yields (60–75%) were from the monocyclic alkaloid, heliotrine, possibly either because of side reactions involving the nortype OH, or because higher water solubility led to greater losses into the aqueous wash.

Attempts to dehydrogenate supinine (h) always led to polymeric products. Evidently dehydrosupinine is too unstable to survive the isolation conditions.

Dehydrogenation of nortype bases (as opposed to ester PAs) required a modified procedure. It was found that the initially formed pyrroles degraded very quickly in the reaction mixture, leading to very impure products in less than 2 min. The solution was to limit the reaction to a very short time at a low temperature. Dehydrotropane (DHR) (l) thus prepared using o-chloramal contained some unchanged retronecine, most of which could be removed by the aqueous wash together with a small amount of product. Nevertheless, a good yield of crystalline DHR was obtained. However if o-bromamal was used, the product was free from starting material, and the residual reagent and its reaction products were easily removed using anion exchange resin, leaving an almost quantitative yield of DHR. Dehydrosupinine (l), being more lipophilic than DHR, could be isolated the same way as dehydro-alkaloids, after an aqueous wash. In contrast, dehydrotropane-
<table>
<thead>
<tr>
<th>Position</th>
<th>Compound</th>
<th>j (ppm)</th>
<th>d (ppm)</th>
<th>1 (ppm)</th>
<th>g (ppm)</th>
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<td>2</td>
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<td>6.31d (3)</td>
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<tr>
<td>3</td>
<td>6.26d (3)</td>
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<td>6.64d (3)</td>
<td>6.65d (3)</td>
<td>6.51d (3)</td>
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<tr>
<td>5</td>
<td>—</td>
<td>ca 4.0 m</td>
<td>ca 4.0 m</td>
<td>ca 4.0 m</td>
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<tr>
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<td>—</td>
<td>1.78d (7)</td>
<td>2.02d (7)</td>
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</table>

*Chemical shifts (δ, ppm) in CDCl₃, 60 MHz, with TMS standard. Coupling constants (J) in parentheses.

cin (g) was extremely water soluble and could not be isolated in the organic phase. Instead, it was prepared using o-bromanil, then extracted into water, leaving bromanil and its products in the chloroform.

Most of the products described were crystalline, and usually practically pure as indicated by spectroscopy and TLC. They could be recrystallized, but rigorous exclusion of moisture was essential in the case of pyrroline esters. The procedures described were ideal for small scale preparations (20 mg or less), but they could be scaled up to 200 mg or more if required. However it is unwise to store large amounts of dehydro-PA as they are liable to decompose—a process which is autocatalytic because one of the products is acidic.

The new products were characterised by their NMR spectra (Table 3), which showed the expected pyrroline signals (H-2, H-3), and signals from the acid moiety similar to, but slightly downfield from those given by the parent alkaloid. Satisfactory mass spectra were obtained using positive ion FAB in a glycerol matrix. Negative ion FAB gave a very strong (M−H) ion from dehydrocrotonetane.

In conclusion, procedures using either o-chloranil or o-bromanil can be recommended for making small amounts of dehydro-PA of excellent purity. Dehydro-nocines are best prepared using o-bromanil; this has enabled DHR to be made quantitatively, and dehydrocrotonetane to be prepared for the first time.

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REFERENCES


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