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A SIMPLIFIED ISOLATION PROCEDURE FOR AZADIRACHTIN

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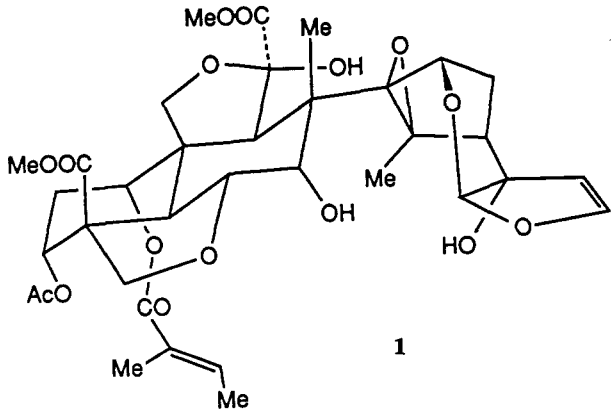
The Indian neem tree, *Azadirachta indica* A. Juss (Meliaceae) has long been recognized for its unique properties. Of primary importance are potent insecticidal properties. Studies (1-15) have revealed an array of bioactive limonoids (16), the seeds being especially well endowed. Further revelation that the action is systemic, the compounds nontoxic, non-mutagenic, and biodegradable, and the wide range of insect species affected (17,18), has made the neem tree the focus of worldwide attention, including three international neem conferences.

Azadirachtin [**1**], the most active component, continues to be studied intensively (17,19,20). The initially proposed 1975 structure (6,7) also has been under scrutiny for several years without success. However, its structure has finally been established (21) including X-ray crystallography of its derivative (22); structure **1** represents the revised structure of this complex limonoid (21-23).



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Several procedures have been published for the isolation of azadirachtin (4-7,12,24). Purification was accomplished by successive partitionings and/or a variety of chromatographic steps including preparative layer chromatography (4-7), open-column reversed-phase chromatography (12) and hplc (24). In spite of these reports, there has been a perennial lack of pure material for research purposes. The great demand for pure azadirachtin has prompted us to publish an improved preparative isolation procedure.

It has been found to be advantageous to exhaustively defat the ground neem seeds with hexane prior to extraction with 95% EtOH (see Figure 1). The ethanolic extract is then subjected to two quick, efficient partitionings: between petroleum ether and 95% aqueous MeOH to remove any remaining oils and other non-polar materials, followed by partitioning between H_2O and EtOAc to remove what appears to be water soluble proteins[1] and sugars. The EtOAc

[1]If the MeOH/H_2O layer from the first partitioning is evaporated to complete dryness (azeotropically with absolute EtOH), a dark, dough-like residue results that is insoluble in absolute EtOH, $CHCl_3$, and EtOAc. The residue is soluble in H_2O or aqueous EtOH. Apparently water soluble proteins have occluded organics.

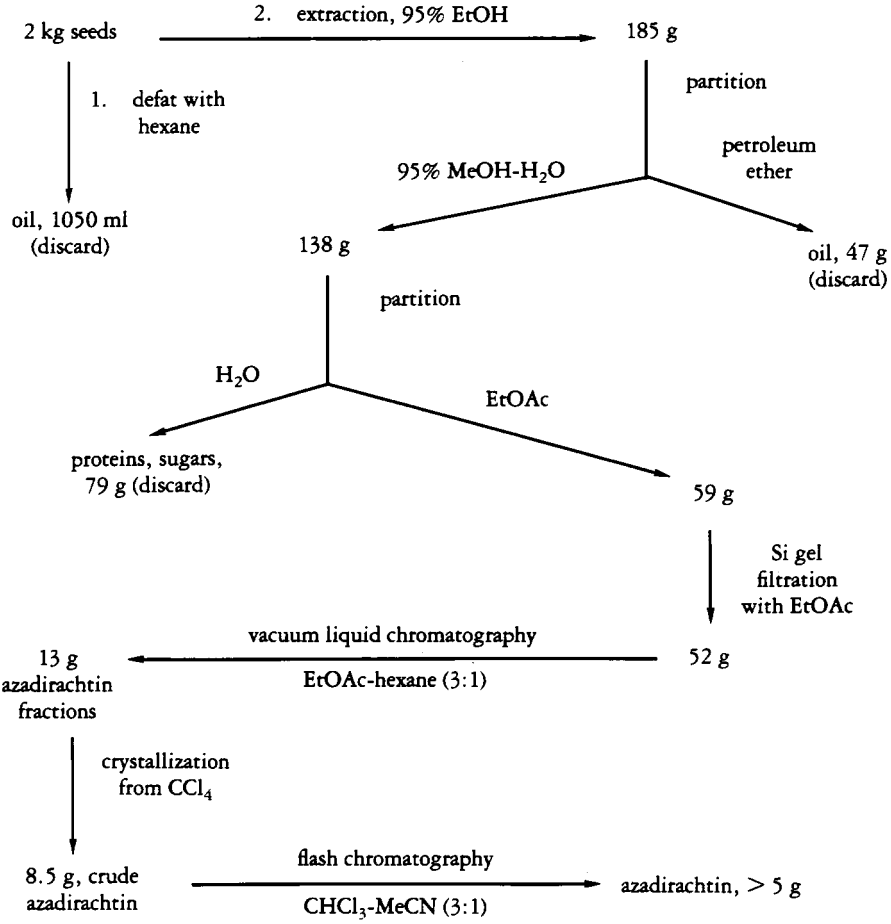

FIGURE 1. Flow diagram for the isolation of azadirachtin from the seed of *Azadirachta indica*.

layer is vacuum filtered[2] through a plug of Si gel (sintered glass funnel) and the filtrate concentrated.

A method was devised to accomplish a large-scale preliminary purification of azadirachtin from the remaining matrix (52 g). A 600-ml sintered-glass funnel packed one-half full with lc grade Si gel (1 cm sand layer) was equilibrated (hexane-EtOAc, 1:3) by applying a vacuum to a filter flask beneath it. The 52 g of material was chromatographed in four portions, and ten 250-ml fractions were collected for each portion.

The first two fractions consisted of a complex mixture of the least polar compounds, presumably other tetranortriterpenoids, by ^{1}H nmr (ca. 20 g). The third fraction was nearly pure salannin (3) in total overall yields comparable to azadirachtin. After a small gap, azadirachtin eluted in fractions 5-8 and could be obtained 70-80% pure at this stage in the main fractions, 13 g total (Figure 1).

Because the remaining impurities were of like polarity and not easily separable on the preparative scale, a crystallization of azadirachtin was carried out. The partially purified material was dissolved in CCl_4 with heating on a steam

[2]Azadirachtin has an Rf=0.7 on Si gel with EtOAc. Attempts to tone down the solvent system with even small amounts of hexane or Et_2O caused precipitation of polar materials, including some azadirachtin. Filtering the EtOAc layer through Si gel at this point removes the interfering polar compounds and renders the filtrate soluble in hexane/EtOAc mixtures for further chromatography.

bath, suction filtered warm, and stored in the freezer. The solution clouded soon after filtering and did not clear for nearly 12 h. An off-white microcrystalline material was collected by suction filtration. Very little, if any, azadirachtin remained in the mother liquor. Analysis of the precipitate revealed that some other more polar materials had co-crystallized, but none of the troublesome compounds of closest polarity were present.

Final purification was accomplished by flash chromatography ($CHCl_3$-MeCN, 3:1), azadirachtin eluting first, essentially pure. ^{1}H-nmr analysis (250 MHz) revealed no detectable impurities. Solvent removal yielded azadirachtin as a clear, colorless glass, or upon further recrystallization from CCl_4 as a white, microcrystalline material (mp 149°). The total yield was in excess of 5 g.[3]

Some further comments are warranted. The exhaustive hexane extraction greatly simplifies subsequent operations. This defatting process might also result in a more efficient ethanolic extraction, thus accounting for the high yields realized in this procedure. Variations in seed content probably exist, but to our knowledge this has not been systematically investigated.

Vacuum liquid chromatography is rapid, reproducible, and offers the advantage that it can easily be scaled up. Another advantage is simplified loading of material and eluting solvents. A similar system has been described elsewhere (25) as "dry-column" flash chromatography, where the funnel is sucked dry after each predetermined volume of eluting solvent. It was also our experience that the separation was not adversely affected, if the glass funnel was sucked dry, as long as the sintered glass funnel was correctly packed and equilibrated initially to prevent channeling. Likewise the flow rate did not noticeably affect the separation. Solvent could be pulled through at an appreciable rate or left to gravity, which was still 20 ml/min.

The key crystallization step from CCl_4 is not an exacting process. In the course of developmental work it was repeated many times. The process was always efficient; the precipitate always microcrystalline. Even material containing 30-40% azadirachtin could be purified in this manner. This time saving step should help provide the large amounts of pure azadirachtin needed for physiological studies.

EXPERIMENTAL

GENERAL METHODS.—Melting points were obtained on a Thomas capillary melting point apparatus and are uncorrected. Tlc was accomplished on 0.25 mm precoated plates of Si gel 60 F-254 (Merck). Visualization was with 3% (w/v) vanillin in absolute EtOH containing 1% (v/v) H_2SO_4 and gentle heating (Rf=0.25, green, $CHCl_3$-MeCN, 3:1). Flash chromatography refers to the low pressure system described by Still (26). Vacuum liquid chromatography (Figure 1) is a modification of the preparative system described by Harwood(25). All preparative separations were on grade 60 Si gel, 230-40 mesh, 60Å (Merck).

EXTRACTION.—Seed kernels from *A. indica* (2.0 kg) were placed in a Waring commercial blender with hexane (2.0 liters) and ground to a fine powder (10 min). After standing 1 h, the mixture was briefly agitated again, and the resulting suspension poured into a large Buchner funnel (16 cm) under vacuum. After solvent removal (discarded), the resulting seed cake was returned to the blender[4] with fresh hexane (2 liters) and the above process repeated a total of four times.

The seed cake was extracted in the same manner with five 2-liter volumes of 95% EtOH. Percolation was again carried out within the blender, except for periods of 8-12 h. The combined EtOH extracts yielded 185 g of a dark, viscous extract after solvent removal in vacuo. Subsequent steps for the extraction of azadirachtin are described above and in Figure 1.

[3]This yield could actually be substantially higher. Only part of the material was purified initially for derivatizations and nmr studies (23). Considerable degradation of azadirachtin in the remaining material occurred upon standing in the solid state in flasks at room temperature. (half-life, ca. 4 months).

[4]An explosion-proof blender should be used because of the possible fire hazard.

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