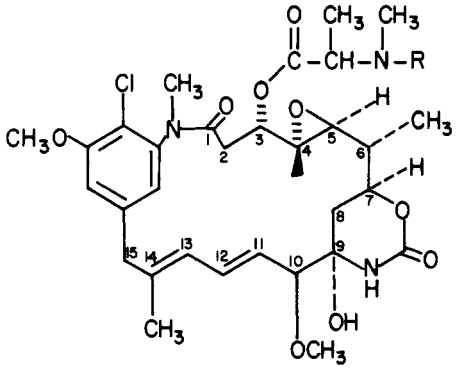
LARGE SCALE ISOLATION OF MAYTANSINE AND OTHER MAYTANSINOIDS FROM THE SEEDS OF *MAYTENUS ROTHIANA* USING PREPARATIVE LC

DONALD E. NETTLETON, JR., DAVID M. BALITZ, MYRON BROWN, JOHN E. MOSELEY and ROBERT W. MYLLYMAKI

Bristol Laboratories, Research Division, Syracuse, N.Y. 13201

ABSTRACT.—A new, highly productive source of maytansine and other maytansinoids has been found in the seeds of *Maytenus rothiana* (Walp.) Lobreau-Callen, a small upland tree of the Western Indian Ghats near Bombay. From this source, a greatly improved and commercially feasible preparative liquid chromatography isolation procedure has been designed. Also, in an off-season collection, the wood of *Maytenus phyllanthoides* Benth (*M. texana* (Benth) Lundell) from the southern Texas Gulf coast has been found to contain only a very small amount of maytansine. In a later collection, no maytansinoids were found in the fruit.

Maytansine (**1**) and other ansamacrolides, including maytanprine (**2**) and maytanbutine (**3**), were first isolated and reported by Kupchan and coworkers (1–6) as being derived from various species of the genera *Maytenus* and *Putterlickia*, both of the family Celastraceae. Shortly after, Wani et al. (7) reported maytansinoids, including maytanbutine, from *Colubrina texensis* Gray (Rhamnaceae). More recently Japanese workers (8) have described the production of ansamitocins, maytansinoids lacking the N-methyl alanyl moiety in the side chain, by fermentations of a *Nocardia* species.



1 R = $COCH_3$, Maytansine

2 R = COC_2H_5, Maytanprine

3 R = $COCH(CH_3)_2$, Maytanbutine

4 R = $COCH_2CH(CH_3)_2$, Maytanvaline

Extensive testing has shown maytansine to be a particularly promising antitumor agent (9), and it is now being clinically evaluated in man.

Unfortunately, work with these compounds has been impeded by the remoteness

of source materials and their exceedingly low maytansinoid content. The ansamitocins may be converted by chemical manipulation to maytansine, but yields are not high. We would like to report a new plant source with high maytansinoid content and a vastly improved and simplified process for the isolation of maytansine and related compounds. We also would like to report the investigation of a potential source of maytansinoids growing in the continental United States.

EXPERIMENTAL

MATERIALS.—Collections of *Maytenus rothiana* were made by Bhogilal C. Shah & Co., Indore, India. The collection of materials from *M. phyllanthoides* was made by one of us (D.E.N.) and Rachel Nettleton. The collection of *M. phyllanthoides* fruit was made by Mr. Paul Carangelo of the University of Texas Marine Science Institute.

EXTRACTION OF THE SEEDS OF *Maytenus rothiana* (WALP) LOBREAU-CALLEN.—One kg of seeds was finely ground in a large blender with a 1:1 mixture of methanol and *iso*-propanol containing 5% water (2 liters). The mixture was stirred for 1 hr and then filtered. Extraction was complete as indicated by the fact that no further amounts of maytansinoids were found (see below for assay procedure) when the spent material was reextracted with fresh solvent mixture.

When the solvents were evaporated *in vacuo* an oily, water-containing residue (about 100 ml) resulted. To this residue was added ethyl acetate (500 ml) and water (200 ml). The mixture was thoroughly shaken, and the two phases were separated. The aqueous phase was extracted two more times with ethyl acetate (100 ml portions).

The combined ethyl acetate extracts were then washed in sequence with 5% sodium hydroxide (200 ml, 4 times), water (100 ml, 4 times), 5% hydrochloric acid (200 ml, 4 times), and portions of water (100 ml, 3 times), according to the method of Kupchan *et al* (4). The ethyl acetate solution was evaporated *in vacuo* to an oil. Dilution with Skellysolve B[1] gave a white flocculant precipitate, which was collected by filtration and dried *in vacuo*; a crude product (2.738 g) was obtained. This material contained maytansine as 5–10% of the uv adsorbing materials (at 254 nm) by hplc analysis as well as other maytansinoids.

EXTRACTION OF SEEDS OF *M. rothiana* (WALP) LOBREAN-CALLEN ON A LARGE SCALE.—The seeds (880 lbs, ~400 kg) were ground in a Ross blender (30 gallon capacity) in 25 kg lots with 50 liter portions of the 1:1 methanol:*iso*-propanol mixture containing 5% water. The alcohol extracts were combined and processed as described above. Crude maytansinoid mixture (1.05 kg) equivalent to that prepared on the laboratory scale was obtained from 880 lbs (~400 kg) of seeds.

BATCH SILICA PURIFICATION OF CRUDE MAYTANSINOIDS.—Silica gel (Woelm, 0.063–0.2 mm-for column chromatography[2], 1 kg) was stirred for 30 minutes with methylene chloride: *iso*-propanol:water (90:10:1) (2 liters) and then with methylene chloride:*iso*-propanol (99:1) (1.2 liters). The crude maytansinoid mixture (400 g) was dissolved in methylene chloride: *iso*-propanol (99:1) (1.2 liters). This solution was stirred with the pretreated silica for 30 min and filtered; the solids were washed with a small amount of the solvent mixture. The silica was stirred for another 30 min with fresh methylene chloride:*iso*-propanol (99:1) and filtered. Evaporation of the combined spent solvent fractions gave a green tar (276 g). The silica was then stirred sequentially with methylene chloride; methanol (60:40), (1.5 liters plus 0.5 liters rinse) and methylene chloride:methanol (1:1) (1.2 liters). From the 60:40 methylene chloride: methanol elution, a yellow-orange semisolid material (53.2 g) which contained the maytansinoids was obtained. The final extraction gave a residue (48.5 g) containing no maytansinoids by hplc.

HPLC ANALYSIS OF MAYTANSINE MIXTURES.—A μ-Porasil[3] analytical silica column, equilibrated with a methylene chloride:*iso*-propanol:water (96:4:0.5) solvent system, was used. The maytansinoids elute in the order maytanbutine (**3**), maytanprine (**2**), and maytansine (**1**) with capacity factors k', respectively, of 1.97, 2.53, and 4.10.[4]

SEMI-PREPARATIVE HPLC PURIFICATION OF CRUDE EXTRACTS OF *M. rothiana*.—A 50 cm by 10 mm (I.D.) column was packed with Partisil 20[5] silica in carbon tetrachloride, using a Haskell

[1]Skelly B, petroleum ether fraction, mainly n-hexane, b.p. 60–68°.
[2]ICN Pharmaceuticals.
[3]Waters Assoc., Milford, Mass.
[4]The capacity factor is defined as the ratio $k' = (k-k_o)/k_o$ where k is the actual retention of the component on the column with any appropriate unit, and k_o is the void or displacement value of the column in the same units.
[5]Whatman, Inc., Clifton, N.J.

pump. The column[6] was equilibrated to a methylene chloride:*iso*-propanol:water (95:5:0.5) system and crude Skellysolve B precipitate (50 mg), obtained as described in the first experiment above, was injected. Following the large frontal peak, four peaks eluted. Also a small shoulder trailed the last. By pmr the last three of these peaks were proved to be, respectively, maytanbutine, maytanprine, and maytansine. The first is suspected to be maytanvaline (4) (Component A); the identity of the shoulder (Component D) is unknown. Data is tabulated (table 1) with fractions taken as guided by the recorder trace (cf fig. 1).

TABLE 1. Data of hplc purification of crude extracts of *M. rothiana*.

Fraction #	Wgt. (mg)[b]	Composition
2–9[a]	17.7	Solvent front mixture
10–11	1.0	Solvent front mixture + 30% unknown component
12–14	0.5	~80% component A (suspected maytanvaline)
15–16	0.2	≥90% maytanbutine
17	0.1	~80% maytanbutine
18	0.1	~40% maytanbutine, ~60% maytanprine
19–20	0.5	≥90% maytanprine
21–23	0.4	mixture
24–25	0.3	~90% maytansine
26–28	1.0	≥95% maytansine
29–31	1.0	~70% maytansine, ~20% late eluting component

[a]Fraction 1 was discarded.
[b]Total weight was 22.8 mg (45%).

PREP LC/SYSTEM 500[7] CHROMATOGRAPHIC PROCESS FOR PURIFICATION (>95% PURITY-MAYTANSINE) OF THE BATCH SILICA TREATED ENRICHED MAYTANSINIOD FRACTION WITH A METHYLENE

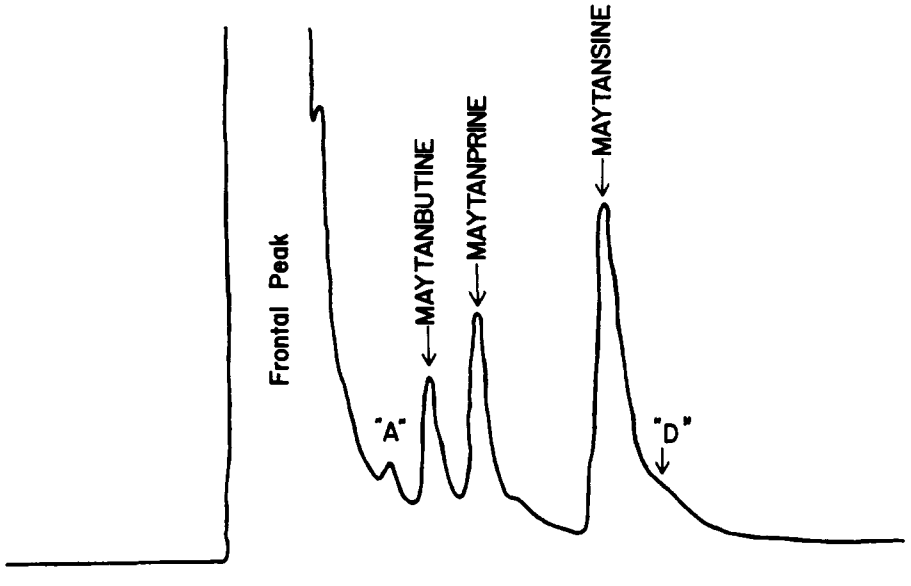


FIG. 1. **M. rothiana seed ext., 50 mg, on Partisil 20, CH_2Cl_2: iso-PrOH: H_2O; (95:5:0.5), 2 ml/min detector at 271 nm, 2.0 AUFS.**

[6]Plate count N = 8130/m as evaluated by the retention and peak width observed for methyl anisate elution in 0.25% *iso*-propanol in *iso*-octane system, flow rate 1.5 ml/min.
[7]Waters Assoc., Milford, Mass.

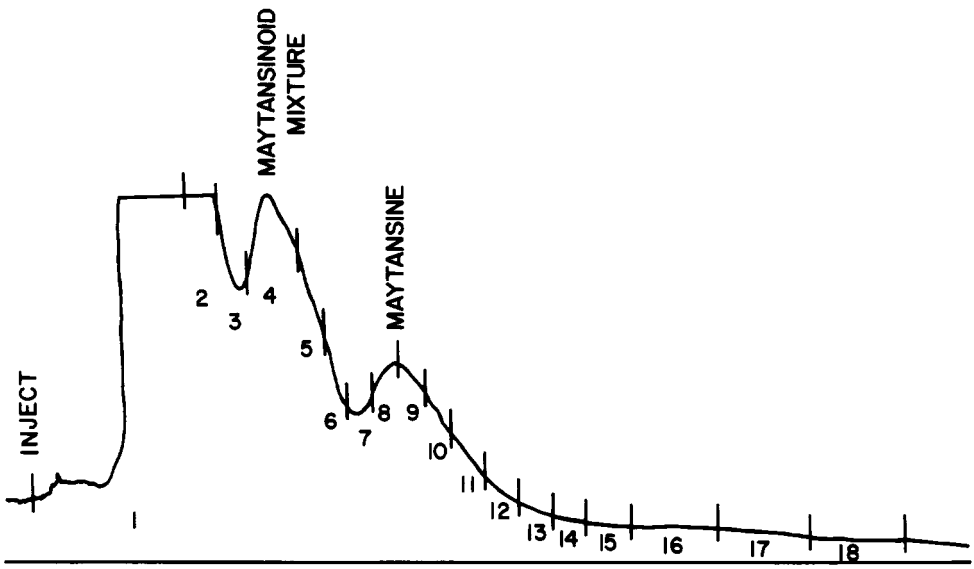


FIG. 2. M. rothiana seed ext., 50 g. Waters Prep LC/500, CH_2Cl_2 : iso-PrOH:H_2O; (95:5:0.5), 200 ml/min., RI detector.

CHLORIDE BASED SOLVENT SYSTEM.—As a routine procedure, newly installed PrepPak silica columns were deactivated by washing them with a solvent that contains a higher alcohol and water content. Typically, the columns were washed with methylene chloride:*iso*-propanol: water content than the actual eluting system. Typically, the columns were washed with methylene chloride:*iso*-propanol:water (75:25:2); the first 4 liters of eluant were passed through and followed by recirculation of solvent until a stable refractive index baseline was obtained. A relative response of 20 and a 200 ml/min flow rate were used.

The solvent system used was methylene chloride:*iso*-propanol:water (95:5:0.5); the mixture was prepared at least 12 hours before use to insure complete phase separation. Care was taken not to draw from the upper water puddle during the run. The PrepPak silica columns were equilibrated to this system; the first 4 liters were wasted to wash out the deactivation solvent; the remaining solvent was then recirculated to a stable refractive index baseline at a relative response of 20.

Crude seed extract (50 g) was dissolved in methylene chloride (150 ml) and pumped onto the columns. Development of the columns was continued with the running solvent at a flow rate of 150 ml/min. At higher flow rates, the large amounts of oil present in the sample caused too high a back pressure. A major frontal band eluted followed by two peaks as seen by the RI detectors (c.f. figure 2). Results are tabulated in table 2.

All fractions taken as shown in table 2 were evaporated to an oil or amorphous, frothy residue. The maytansine-rich fraction was taken up in methylene chloride and reduced in volume on a steam bath. Warm diethyl ether was added as volume reduction was continued with care being taken to avoid bumping. After several additions, crystallization began and the mixture was cooled slowly, first to ambient temperature and then to −20°. In some early experiments, seeding was used to induce crystallization. The solids were collected and dried to give maytansine, 1.71 g, identical by pmr, ir and other properties to that described in the literature (5). Maytansine-rich fractions in yields of up to 9.8% based on starting weight have been realized from 75 g runs. Crystallization yields are generally around 40% from the amorphous dried fraction or about 4% overall.

CRYSTALLIZATION OF MAYTANPRINE.—The oily residue (3.9 g) from the maytanprine-rich fractions from several preparative lc chromatograms was dissolved in methylene chloride, 50 ml, and treated as described for maytansine in the preceding experiments. The white crystalline product, 382 mg, was identical by pmr and other spectral data to maytanprine described in the literature (5).

TABLE 2. Results of liquid chromatography of crude seed extract.

Cut #	Elution vol.[a]	K (Range)[a]	Wgt.	Composition (uv adsorbing compds.)
1–2	0–1.5	0–0.56	—	Discarded, frontal peak
3	1.5–1.86	0.56–1.33	3.08 g	Frontal (plus early maytansinoids?)
4	1.86–2.07	1.33–1.59	1.90 g	Mostly maytanbutine
5	2.07–2.30	1.59–1.88	2.10 g	1:1 maytanbutine:maytanprine
6	2.30–2.50	1.88–2.13	1.33 g	1:4 maytanbutine:maytanprine
7	2.50–2.70	2.13–2.38	0.92 g	~90% maytanprine
8	2.70–2.90	2.38–2.63	0.78 g	1:1 maytanprine:maytansine
9–14	2.90–4.55	2.63–4.69	**	>95% maytansine
15	4.55–4.85	4.69–5.06	0.24 g	Maytansine plus late material
16–21	4.85–9.22	5.06–10.5	2.81 g	Post maytansine components

[a]Volumes are in liters, and the ranges given are the total volumes of solvent eluted after injection of the sample as determined at the start and at the end of collection of any particular cut. The actual volume of the cut is the difference in the two values. The capacity factors, K, are calculated here from the equation $K=(V-V_o)/V_o$ where V is the volume and V_o the void volume (c.f. footnote 4). For the Prep 500 apparatus with two columns in the chamber, V_o effectively equals 0.8 liter.

**This sample was crystallized directly on work up; maytansine of $\geq$95% purity was obtained.

CRYSTALLIZATION OF MAYTANBUTINE.—An oily maytanbutine-rich fraction, 1.196 g, from Prep 500 runs was treated as the other maytansinoids were in the previous two experiments to give a white crystalline product, 76 mg, identical by pmr to maytanbutine described by Kupchan et al. (5).

PREP LC/SYSTEM 500 CHROMATOGRAPHIC PROCESS FOR PURIFICATION OF MAYTANSINE WITH AN ETHYL ACETATE BASED SOLVENT SYSTEM.—Two PrepPak silica columns which had been previously used for maytansine purifications with the methylene chloride based system were washed with ethyl acetate (4 liters) and then pre-equilibrated with an ethyl acetate:*iso*-propanol (90:10) system (8 liters); half the volume was pumped to waste and the remainder was circulated as described above. The columns were then equilibrated to the running system, ethyl acetate:*iso*-propanol (95:5). Maytansine (7.6 g), previously chromatogrammed in the methylene chloride based system, was dissolved in the running solvent (125 ml), filtered, and pumped on to the columns. Development was carried out at 200 ml/min.

Two peaks eluted as shown by the RI detector. The second was the major peak and, by tlc analysis, consisted of pure maytansine. Tlc analysis was carried out on Analtech silica gel GF plates with 10:1 ethyl acetate:methanol system. Results are tabulated in table 3.

TABLE 3. Results of liquid chromatographic purification of maytansine.

Cut #	Elution vol.[a]	K (range)	Wgt. yield	Nature
1	0–1.25	0–0.56	0.066 g	Frontal material, undefined
2	1.25–2.06	0.56–1.58	0.751 g	Mainly premaytansine impurity (by tlc)
3	2.06–2.19	1.58–1.74	1.093 g	90% maytansine
4	2.19–3.6	1.74–3.5	5.179 g	100% maytansine

[a]In liters, c.f. table 2 for explanation of elution vol. and K.

COLLECTION, EXTRACTION, AND ANALYSIS OF *Maytenus phyllanthiodes* BENTH. (*M. texana* (BENTH) LUNDELL).—Specimens of aerial parts (leaves, twigs and small branches) were collected on February 3, 1979, from clay bluffs some 50–100 ft north of Texas route 4 at a point about 15.5 miles east of Brownsville, Texas. After 8 hrs the material still appeared fresh. Leaves were separated from twigs, bark, and branches; larger branches were split, and the materials were placed in plastic bags in a refrigerator. The materials were shipped to our laboratories by air express and stored frozen until they could be extracted.

The separated parts were each extracted by the procedure used for *M. rothiana* seeds including the basic and acid washing steps. From 480 g of twigs, bark, and wood (branches) a light green powder, 75.2 mg, was obtained from Skellysolve B precipitation. By the an-

alytical hplc process described above this precipitate appeared to contain a small amount of maytansine ($\leq 1\%$), the peak being enhanced by spiking with an authentic sample of the ansamacrolide. The leaves, 90 g, afforded a powdery green solid, 362 mg, which by hplc analysis contained no maytansine.

EXTRACTION AND ANALYSIS OF *M. phyllanthoides* FRUIT.—Specimens were collected in the same area as in the preceding experiment on June 23, 1979, by Mr. Paul Carangelo. The material, 183 g, was extracted and processed in the same manner to give a Skellysolve B precipitate, 285 mg, showing no maytansine content by hplc analysis.

RESULTS AND DISCUSSION

The fruits of *Maytenus rothiana* (Walp.) Lobreau-Callen, a small upland tree from the Western Ghats of India in an area east and south of Bombay, were first collected in the course of a National Cancer Institute (NCI) search for better sources of maytansine. Extracts of these were found to possess considerable antitumor activity, and subsequently a large collection was made of husks and seeds separately. The materials arrived at the time Bristol Labs first became involved with maytansinoids, and were, therefore, sent to us for evaluation and extraction.

The existent isolation procedures were too tedious and involved for either chemical analysis or isolation on a commercial scale. Accordingly, the first undertaking was to design an HPLC analytical system using reference maytansine provided by the NCI. With methylene chloride as the carrier solvent and *iso*-propanol as polar modifier, good retention of maytansinoids was observed on silica, but band spread and tailing were serious.

In earlier work with anthracyclines having aminoglycoside moieties (10), we had experienced similar problems due in part to amino function interactions with acidic sites on the silica packings. Inclusion of a base in the solvent system was sufficient to neutralize the acid sites, but good resolution with sharp peaks was only achieved when small amounts of water were also added. In the case of the maytansinoids, being neutral compounds, addition of bases would be expected to have little specific value, but water could have the same effect as with the anthracyclines.

As anticipated, the addition of 0.5% water to a 96:4 methylene chloride: *iso*-propanol system gave dramatic improvement in the resolution. Both analytical, and one semi-preparative, runs on silica packings gave the same pattern (cf fig. 1) with three major maytansinoid peaks eluting, maytansine being the last. The elution sequence is in order of decreasing lipophilicity due to differences in the acyl moiety of the side chain.

The second and third peaks, after the large frontal peak and excluding shoulders, have been found to be, respectively, maytanbutine and maytanprine by pmr analysis of various fractions accumulated from preparative work. The identity of these compounds and that of maytanine, were rigorously established by comparison to pmr and infra-red data published by S.M. Kupchan and co-workers (6). The small first peak, labeled A in figure 1, is probably maytanvaline, but yields and purity of this fraction were not good enough to permit definite identification.

The translation of the analytical hplc process to semi-preparative and preparative scale work went smoothly. Because Partisil 20 and the PrepPak silica cartridges tend to be more active than μ-Porasil, on which most of the analytical work was done, the polar modifier was increased by 1%. Loads of up to 75 g crude material, enriched by batch silica treatment, could be made with excellent resolution of maytansine as shown in figure 2. With lighter loads such as described

in the experimental section, the RI detector on the Prep 500/LC system was too insensitive to show any but the frontal peak. In all cases fractions were analyzed by hplc before pooling and work-up.

Despite the appearance of good fractionation, maytansines obtained from the Prep 500 runs were always heavily contaminated with oils. Unlike the maytansinoids, these apparently streak and tail badly even when light loads are applied to the columns. Fortunately they are removed by crystallization.

Analysis of maytansine submitted to the National Cancer Institute by the Life Sciences Division of SRI International showed a faster moving impurity resolved by tlc in a 5% methanol in ethyl acetate system. This could not be separated or detected in the methylene chloride lc systems used previously, even with extensive recycling. The impurity was separated, however, in an ethyl acetate based system on the Prep 500 apparatus, as described in the experimental section. *Iso*-propanol was again better than methanol as the polar modifier.

The initial extraction as well as acid and base washes follow the procedure described in the maytansine patent (4). In early work 95% ethanol was used, but, due to the various federal regulations and restrictions involved, even for denatured alcohol, an alternative was sought for large scale extraction in the pilot plant. For this purpose methanol-*iso*-propanol (1:1) containing 5% water was at least equivalent to the ethanol system, if not superior. Methanol, *n*-propanol, *iso*-propanol and higher alcohols with 5% water were inferior.

The crude solids precipitated by Skellysolve B after the acid and base washes still contain a considerable amount of oily contaminants. The batch process described in the experimental section was devised to simplify the mixture before final chromatography. This has also improved our ability to detect maytansinoids in various plant materials sent to us for evaluation, particularly where there is sufficient oil to inhibit precipitation.

The collection and analysis of leaves and branches of *Maytenus phyllanthoides* Benth, or *M. texana* (Benth) Lundell, as specimens placed in Texas herbariums are generally named, arose from opportunity and from curiosity as to whether the only *Maytenus* species reported for the continential United States would contain these interesting agents. While the Indian source is probably the best found to date, it also seemed worthwhile to establish the potential of additional sources within this country. Wani et al. (10) have already reported maytansinoid metabolites, including maytanbutine, from *Colubrina texensis* Gray (Rhamnaceae).

M. phyllanthoides, known commonly as leatherleaf of guttapercha maytenus, is a decumbent evergreen shrub. Erect specimens growing to the size of small trees have been reported but not observed by us. It is a coastal plant reported from the lower Florida peninsula and Keys, the lower Texas Gulf coast, and the lower California coast. In Texas, it is found growing on clay dunes, although it has been observed in other types of soil.

Our collection was made in early February and, although the flowering period reported is April to November, a few plants displayed several small flower clusters. An additional fruit collection was made in June. Analysis showed what may be small amounts of maytansine in the woody fraction but none in the leaves or fruit. Confirmation of maytansine in the wood would require a much larger collection and isolation of the metabolite, an effort which we did not have time to carry out.

Since the highest concentration of maytansine reported to date is from a seed fraction, the possibility arises that maytansine has some function within that part.

Generally it has been found otherwise in woody fractions where it may well have been deposited during plant cycles and, being reasonably stable, had persisted. Two Japanese workers have reported antimitotic, antigibberellin, and auxin activities for the compound (11), which indicates some growth regulatory potential for it. If maytansinoids do have some function in the seed, collection of this part from other species may be of value.

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