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ALIPHATIC NITRO COMPOUNDS FROM *ASTRAGALUS* SPECIES*

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Abstract—Miserotoxin, the poisonous constituent of *Astragalus miser*, has been shown to be 3-nitro-1-propyl β-D-glucopyranoside. From *A. cibarius*, 3-nitropropanoic acid was the major isolate from an ethanol extraction, but a diester of 3-nitropropanoic acid with glucose was the major product from an acetone extraction. From a small plant sample of *A. curvicarpus*, 3-nitropropanoic acid and KNO_3 were isolated. Minor non-nitro compounds were also isolated from *A. cibarius*, including (for the first reported time as a natural product) *N*,*N*-dimethylformamide.

INTRODUCTION

LIVESTOCK poisoning due to the ingestion of various *Astragalus* (Leguminosae) species (locoweeds, poison vetches) has been known for many years. These plants have been divided[1] into groups based upon three main types of toxicity: (1) selenium poisoning caused by a group of selenium accumulators, (2) the chronic true 'loco' symptoms appearing particularly in horses, and (3) an acute poisoning caused by several species and typified by that due to *A. miser* varieties (timber milkvetches). We undertook study of a particularly toxic *A. miser* variety and, upon establishing that the toxic properties were due to an aliphatic nitro compound, then investigated the nitro compound content of some other *Astragalus* species.

RESULTS

Astragalus miser *Dougl. var.* oblongifolius *(Rydb.) Cronq.*

As described in the Experimental, we isolated from this plant (+)-pinitol, a flavonoid shown[2] to be 4′-*O*-methylquercetin-3-glucoside, and a poisonous compound which we dubbed miserotoxin.[3]

Miserotoxin was a homogeneous, optically active ($[\alpha]_D^{25}$ −22° (c 2·0, H_2O)) oil which resisted all attempts at crystallization, but gave a complex, reproducible NMR spectrum

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† National Aeronautics and Space Administration Predoctoral Fellow.

[1] J. M. KINGSBURY, *Poisonous Plants of the United States and Canada*, p. 306, Prentice-Hall, Englewood Cliffs, New Jersey (1964).

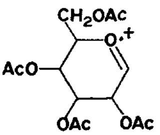
[2] F. A. NORRIS and F. R. STERMITZ, *Phytochem.* **9**, 229 (1970).

[3] See F. R. STERMITZ, F. A. NORRIS and M. C. WILLIAMS, *J. Am. Chem. Soc.* **91**, 4599 (1969); for a preliminary report on the structure of miserotoxin.

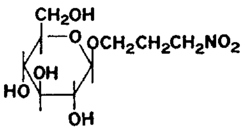
which did not change after repeated purifications. Combustion analysis was consistent with an empirical formula of $C_9H_{17}NO_8$. The mass spectrum of miserotoxin did not show a molecular ion corresponding to this formula. Prominent in the spectrum, however, was a fragment at *m/e* 88·03806 (88·03986 calc. for $C_3H_6NO_2$)[4] and other fragments showed the presence of one nitrogen. Of various tests performed to establish the nature of the nitrogen function, only the Griess-Ilosvay[5] and 'red, white, and blue'[6] tests were positive. These indicated that miserotoxin contained a primary nitro group. The $C_3H_6NO_2$ mass spectral fragment could then be logically assigned to $(CH_2CH_2CH_2NO_2)^+$. In confirmation, the IR spectrum of miserotoxin showed prominent bands at 1550 and 1380 cm^{-1}, indicative of a primary nitro group.[7] The IR spectrum also showed strong OH absorption and the presence of OH was apparent from the NMR. The physical behavior, functionality and empirical formula of miserotoxin suggested that it was carbohydrate in nature and this was confirmed by standard color tests. Miserotoxin was therefore acetylated, but the NMR of peracetylmiserotoxin indicated the presence of *six* CH_3CO groups. This was confirmed by mass spectrum[4] which showed a molecular ion at 519·1583, corresponding to $C_{21}H_{29}NO_{14}$. Combustion analysis of the non-crystalline peracetyl derivative confirmed this empirical formula. This anomalous result will be discussed below. Trimethylsilylation of miserotoxin yielded a derivative which showed the introduction of only four trimethylsilyl groups (mol. wt. = 555 by low resolution mass spectrum) and confirmed the $C_9H_{17}NO_8$ formulation for miserotoxin.

Miserotoxin was hydrolysed with dilute HCl and the hydrolysate was separated into an ether soluble and a water soluble layer. From the ether layer was isolated a yellow oil whose NMR and IR spectra were consistent with its formulation as 3-nitro-1-propanol and this was confirmed by comparison with an authentic sample synthesized from $AgNO_2$ and 3-bromo-1-propanol. From the aqueous layer was isolated a sugar which was shown to be glucose by TLC and GLC of its trimethylsilyl ether. The NMR spectrum of a known 1 : 1 mixture of 3-nitro-1-propanol and glucose in dilute HCl and that of the total miserotoxin hydrolysate were virtually identical.

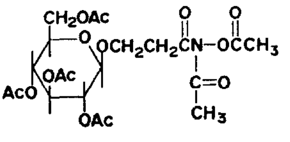
The position of attachment of the 3-nitro-1-propanol to glucose was established at the anomeric carbon since miserotoxin was not a reducing sugar and since the peracetyl miserotoxin exhibited its most intense mass spectral fragment between *m/e* 120 and 519 at *m/e* 331. This peak is attributable to I. The NMR spectrum of miserotoxin in D_2O (after repeated evaporation from D_2O solution) showed a one proton doublet ($J = 7·5$ c/s) at 4·40δ, which is characteristic of the anomeric α proton of a pyranose sugar and hence miserotoxin should be a β-glucoside. Periodic acid oxidation of miserotoxin yielded 0·9 eq/mole of formic



(I)



(II) Miserotoxin



(III)

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[4] High-resolution spectrum from the Purdue Mass Spectrometry Center, supported by U.S. Public Health Service grant RF-00354.

[5] P. Bose, *Analyst* **56,** 504 (1931).

[6] A. I. Vogel, *Elementary Practical Organic Chemistry* (3rd Edition), p. 537, Wiley, New York, (1957).

[7] R. M. Silverstein and G. C. Bassler, *Spectrometric Identification of Organic Compounds* (2nd Edition), p. 99, Wiley, New York (1967).

acid which is consistent with the glucopyranose structure and not with a glucofuranose. Thus, miserotoxin can with confidence be assigned structure II, 3-nitro-1-propyl β-D-gluco-pyranoside.

Some parts of the NMR spectrum are worthy of discussion. Of particular value in in analysing fractions by NMR for the presence of miserotoxin was the quintet ($ROCH_2C\underline{H}_2CH_2NO_2$) appearing at 2·30δ, a part of the spectrum devoid of other absorptions. The characteristic triplet at 4·64δ ($ROCH_2CH_2C\underline{H}_2NO_2$) was blocked out by the HDO peak with miserotoxin in D_2O. Addition of a drop of DCl moved the HDO peak down-field, but interference was still observed from the anomeric proton doublet. However, a re-evaporation of miserotoxin in D_2O several times before finally taking a spectrum gave the best result and separated the triplet and doublet as well as removed the HDO interference. Both this triplet and the quintet from the internal CH_2 are fortuitous since the protons involved are diastereotopic. The groups in question are far enough away from the optically-active center so that the protons are magnetically equivalent. No direct assignment could be made for the remaining CH_2 ($ROCH_2CH_2CH_2NO_2$). There is definitely no triplet present in the proper position and hence the diastereotopic nature of these protons does result in magnetic non-equivalence.

The anomalous acetylation reaction of miserotoxin remained to be explained. The incorporation of six $CH_3C{=}O$ functions into structure II would mean that four were normal acetylations of the ring OH groups, while the remaining two must have been the result of reaction at the NO_2 function. That this was indeed so was shown by performing similar acetylations of nitroethane, nitropropane and other derivatives.[8] On the basis of these reactions, as well as elemental analysis, mass spectrum and NMR, peracetylmiserotoxin could be assigned structure III.

Miserotoxin appeared to be the only nitro compound present in *A. miser* var. *oblongifolius* as all fractions (from numerous separation and isolation methods) which were toxic or showed positive Griess-Ilosvay tests for nitro compounds proved to contain only miserotoxin. Reports have been previously made on the distribution of miserotoxin in other varieties of *A. miser*,[9] on toxicity[10] and on metabolism.[11] The metabolism and toxicity studies indicated that in some species the major toxicity arises from acute nitrite poisoning (3-nitro-1-propanol being metabolized to yield NO_2^-), but that in others (e.g. cattle) death could occur without the typical symptoms of nitrite poisoning. In the latter case, 3-nitro-1-propanol was found to be rapidly formed by miserotoxin hydrolysis in the rumen and subsequently transported to the brain where it affected the sites controlling respiratory and muscular responses.[10,11]

Astragalus cibarius *Sheld.*

In the course of field testing various *Astragalus* species for the presence of nitro compounds, it was discovered that *A. cibarius* gave a strong positive test. *A. cibarius* is known as the 'browse milk-vetch' and is described[12] as being 'suitable for fodder'. An ethanol

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[8] F. R. Stermitz and F. A. Norris, *J. Org. Chem.* **35**, 527 (1970); F. R. Stermitz and D. W. Neiswander, unpublished results.

[9] M. C. Williams and F. A. Norris, *Weed Sci.* **17**, 236 (1969).

[10] M. C. Williams and W. Binns, *Weeds* **15**, 359 (1967); M. C. Williams, K. R. Van Kampen and F. A. Norris, *Am. J. Vet. Res.* **30**, 2185 (1969).

[11] M. C. Williams, F. A. Norris and K. R. Van Kampen, *Am. J. Vet. Res.* **31**, 259 (1970).

[12] R. C. Barneby, *Atlas of North American Astragalus*, The New York Botanical Garden, Bronx, New York (1964).

extract of this plant yielded (+)-pinitol, succinic acid, malonic acid, an isorhamnetin-3-*O*-glycoside, *N,N*-dimethylformamide, 3-nitropropanoic acid, ethyl 3-nitropropanoate, and a small amount of a yellow, non-crystalline semi-solid nitro compound which has resisted complete characterization.[13]

We could not find a previous report in the literature on the natural isolation of *N,N*-dimethylformamide (DMF). Because DMF is a common solvent, we checked all solvents and other materials used in the isolation as well as collection of the plant and could find no avenue of contamination. Use of an acetone extraction of the plant material in place of ethanol still resulted in the isolation of DMF. Again, the acetone solvent itself was free of DMF. We plan to confirm this first report of DMF as a natural product by checking greenhouse-grown *A. cibarius* and by seeking to establish its presence in other *Astragalus* species.

Of major interest is the finding that the nitro compounds of *A. cibarius* are based on 3-nitropropanoic acid rather than 3-nitro-1-propanol as in *A. miser*. This difference accounts for the decreased toxicity of *A. cibarius* as it has been shown[10] that no toxic symptoms were apparent in cattle when 3-nitropropanoic acid was fed at the 50 mg NO_2/kg level, whereas 3-nitro-1-propanol was fatal at the 25 mg NO_2/kg level. Ethyl 3-nitropropanoate appears to be an artifact since it was not isolated in a separation scheme employing anhydrous acetone as the first extractant instead of ethanol, but in which all other steps remained the same. The acetone procedure, in addition, yielded far less 3-nitropropanoic acid and far more of the presumed glucose diester than did the ethanol procedure.

Astragalus curvicarpus *(Heller) Macbr. var.* curvicarpus

Field testing of this plant gave a positive indication for nitro compounds. Since the amount of plant material available was small, only major constituents could be determined. A precipitate appeared in the original ethanol extract. This was filtered off and found to be potassium nitrate. Work-up of the extract yielded crystalline (+)-pinitol and 3-nitropropanoic acid. No information was secured on the original state of the 3-nitropropanoic acid in the plant since the only material available was extracted via the ethanol procedure which was later to be capable of hydrolysing 3-nitropropanoic acid esters. No 3-nitropropanol derivatives were found and these are known to survive the ethanol extraction procedure.

Phytochemical Survey

The Griess-Ilovsay test for nitroalkanes[14] is sufficiently sensitive so that a 1–2 mm plant stem portion and a small leaf or two provide sufficient material for testing. Hence, testing of herbarium specimens which contained redundant material was possible. A total of 116 voucher specimens in the Colorado State University herbarium were tested and these represented about 100 species or varieties. Table 1 lists the name, location, collection date, and CSU herbarium accession number of those specimens giving positive tests. We also performed Griess-Ilosvay tests on some 70 different plant species picked at random in the vicinity of Fort Collins, Colorado. None of these was positive.

[13] Preliminary data suggest that this compound is a diester of 3-nitropropanoic acid with glucose, probably the 1,6-diester.

[14] This test is also positive for inorganic nitrite, an occasionally found plant constituent.

TABLE 1. *Astragalus* SPECIES GIVING A POSITIVE TEST FOR NO_2 COMPOUNDS

Plant	Location	Collection date	Accession No.
A. aboriginum Rich.	Grand Co., Colo.	1962	42001
A. aequalis Clokey	Clark Co., Nev.	1937	12399
A. canadensis Mortoni	Almota, Wash.	1894	12448
A. caryocarpus Ker.	Dickinson Co., Iowa	1912	36822
A. ceramicus Sheld.	Montrose Co., Colo.	1967	46090
A. cibarius Sheld.	Evanston, Wyo.	1898	12423
A. coltoni Jones var. *moabensis*	Mesa Co., Colo.	1947	26749
A. diversifolius Gray	Moffat Co., Colo.	1959	23970
A. nuttallianus D.C.	San Juan Co., Utah	1957	37082
A. scopulorum Port.	San Miguel Co., Colo.	1967	46041
A. sericoleucus Gray	Sioux Co., Nebr.	1956	39604
A. spatulatus Sheld.	Sioux Co., Nebr.	1956	39597
A. subcinereus Gray	Navajo Co., Ariz.	1961	41203
A. tenellus Pursh.	White River, Colo.	1947	35485
A. toanus Jones	Owyhee Co., Idaho	1946	35486

DISCUSSION

Previously known nitroaliphatics from higher plants are (1) 3-nitropropanoic acid or its glucose derivatives from *Hiptage benghalensis*, *Corynocarpus levigata*, *Indigofera endecaphylla* and *Viola odorato* and (2) 1-nitro-2-phenylethane from *Ocotea pretrosa* and *Aniba canelilla*. The former additionally appears widespread as a constituent of microorganisms. The chemistry, distribution and biosynthesis of natural nitro compounds has recently been reviewed.[15]

In addition to the importance of this work in relation to livestock poisoning.[1,9–11] the isolation of a 3-nitro-1-propanol derivative provides the first structural relative of the widely-distributed 3-nitropropanoic acid. Since our work has shown that both nitro compounds occur in *Astragalus* species, a biosynthetic connection between the two may exist. Labelled 3-nitropropanoic acid can be fed to *A. miser* and labelled 3-nitro-1-propanol to *A. cibarius* to check for interconversions. Both species are presently being grown in the greenhouse and we contemplate studying this relationship as well as the ultimate origin of the NO_2 function. This is of importance since a preliminary study[16] in the greenhouse has indicated that nitrate fertilizers may be more effective than ammonium fertilizers in increasing miserotoxin levels in the plant. This would be opposite to what would be expected based upon biosynthetic work done on 3-nitropropanoic acid in microorganisms where the path is from NH_4^+ to amino acids to nitropropanoate. However, metabolic differences between higher plants and microorganisms are often observed.[17] In any event, the preliminary result remains that high nitrate apparently causes an increase in production of a toxic organic nitro compound and this result is important in considering the effects of nitrate 'pollution'.[20]

Our herbarium testing results indicate that nitro compounds may be present in about 15% of the *Astragalus* species. On the basis of our preliminary random screening tests, such

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[15] L. M. LARSON, *Dansk. Tidsskr. Farm.* **42,** 272 (1968).

[16] M. C. WILLIAMS, unpublished results.

[17] Indeed, it has been shown[18] that aspartic acid is an important precursor of 3-nitropropanoic acid in *Penicillium atrovenetum* while it was not a precursor of 3-nitropropanoic acid in *Indigofera spicata.*[19]

[18] P. D. SHAW and S. A. MCCLOSKEY, *Biochem.* **6,** 2247 (1967).

[19] E. CANDLISH, L. J. LACROIX and A. M. UNRAU, *Biochem.* **8,** 182 (1969).

[20] H. A. PAINTER, *Water Research* **4,** 393 (1970); D. R. KEENEY, *J. Milk Food Tech.* **33,** 425 (1970).

an incidence does not appear likely for higher plants in general. Since another major source of nitro compounds is in another legume species (*Indigofera endecaphylla*), we plan to shortly screen other legumes in order to establish the incidence of nitro compounds in the family as a whole.

EXPERIMENTAL

Plants of *Astragalus miser* Dougl. var. *oblongifolius* (Rydb.) Cronq. were collected in June 1968, 17 miles southeast of Logan, Utah at 2000 m elevation and included voucher sample No. 120626, deposited in the Intermountain Herbarium, Utah State University. The collections of *A. cibarius* Sheld. were made 5 miles north of Logan in April and May 1969 at 1500 m elevation and vouchers were deposited under numbers 123404 and 123405 at the Intermountain Herbarium and numbers 49033 and 49034 at the Colorado State University herbarium. Collection of *A. curvicarpus* (Heller) Macbr. var. *curvicarpus*[21] was made 11 miles south of Paisley in Lake County, Oregon in July 1970 and a voucher sample (No. 49276) was deposited in the Colorado State University herbarium. The cleaned plant material was loosely packed in burlap bags and dried in a forced air oven at 60° for 48 hr. It was then ground and stored in sealed plastic bags.

A. miser *extraction* (*isolation and identification of miserotoxin*). Dry ground plant material (338 g) was extracted with ethanol in a Soxhlet for 24 hr, cooled to room temp. and filtered. The ethanol solution was evaporated and the green residue taken up in 300 ml H_2O and extracted with $CHCl_3$ and then with *n*-BuOH. The aqueous layer was then evaporated to yield 75 g of a toxic[22] reddish-brown syrup. This was taken up in butanol saturated water and purified by use of an H.O. Post Model B-5 250 tube automatic counter current distributor (upper phase was water satd. butanol and the lower phase was butanol satd. water). Analysis of the fractions was by bioassay, Griess-Ilosvay test,[14] NMR, and TLC (R_f 0·43 in *n*-BuOH–HOAc–H_2O). A total of 8·8 g (2·6% of pure miserotoxin was isolated as a yellow syrup). Anal.: C, 40·5; H, 6·14; N, 5·06. Calcd. for $C_9H_{17}NO_8$: C, 40·3; H, 6·80; N, 5·21%.

The only fractions showing positive Griess-Ilosvay tests also contained miserotoxin as the only nitro compound and were the only fractions giving positive bioassays for toxicity.

The following spectral data were obtained for miserotoxin:

NMR (D_2O after several evaporations from D_2O). 2·30 (quintet, 2 H, J = 6 c/s, $CH_2C\underline{H}_2CH_2NO_2$) 3·0–4·1 (multiplets, 9 H, ring protons and $OC\underline{H}_2CH_2CH_2NO_2$) 4·40 (doublet, 1 H, J = 7·5 c/s, anomeric α proton of a pyranose sugar) 4·64 (triplet, 2 H, J = 6 c/s, $CH_2CH_2C\underline{H}_2NO_2$)

IR (neat): 3400 (strong, broad, OH) 1550 (strong, sharp, NO_2) 1380 (medium, sharp, NO_2) 1050 (strong, broad, C–O)

Optical rotation. $[\alpha]_D^{25}$ −22·8° (*c* 4·7, H_2O)

Mass spectrum.[4] Only minor peaks above *m*/*e* 100. *m*/*e* 88·03806, rel. int. = 50 (calc. for $C_3H_6NO_2$: 88·03986).

Peracetylmiserotoxin (III). Miserotoxin (0·365 g, 1·36 m-mole) and freshly fused NaOAc (0·56 g, 6·8 m-mole) were suspended in Ac_2O (30 ml) and the resulting mixture was heated for 4 hr at *ca.* 80°. The reaction mixture was cooled to 25° and poured into ice water. The heavy oily product was separated from the aqueous layer and the aqueous layer was extracted several times with $CHCl_3$. These $CHCl_3$ solutions were dried, evaporated, and the residue combined with the oily product. Together they were dried at reduced pressure over P_2O_5. In spite of repeated attempts at crystallization, the product remained an oil. *Anal*: C, 48·6; H, 5·60; N, 2·70. Calcd. for $C_{21}H_{29}NO_{14}$: C, 48·06; H, 5·90; N, 2·69%.

Mass spectrum.[4] *m*/*e* 519·1583 ($C_{21}H_{29}NO_{14}$ = 519·1588) 403·1265 ($C_{17}H_{23}O_{11}$ = 403·1240, assigned to M^+–NAc(OAc)) 331·1028 ($C_{14}H_{19}O_9$ = 331·1029, assigned to M^+–$HOCH_2CH_2CONAc(OAc)$). These three peaks were the most prominent in the mass spectrum above *m*/*e* 242. Other spectral data are reported in a thesis.[23]

Tetratrimethylsilyl miserotoxin. Miserotoxin (50 mg) was taken up in dry pyridine (20 ml) and to this was added 1 ml each of 1,1,1,3,3,3-hexamethyldisilazane and chlorotrimethylsilane. The solvent and excess reagents were immediately removed *in vacuo* and the residue was taken up in dry CCl_4 and filtered. Evaporation of the CCl_4 left a viscous residue whose mass spectrum showed M^+ at *m*/*e* 555, consistent with a tetratrimethylsilyl derivative of II.

Periodic acid oxidation. To a solution of miserotoxin (0·192 g in 50 ml H_2O) was added 25 ml of 0·05 N periodic acid. The solution was allowed to stand 8 hr at 25°. The mixture was then titrated with standard base. Titration showed the presence of 0·90 eq/m of acid formed.

Acid hydrolysis. Miserotoxin (1·12 g) was taken up in 2 N HCl (12 ml) and heated on a steam bath for 4 hr in N_2. The solution was then extracted continuously with ether for 12 hr. The ether solution was dried

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[21] We are indebted to R. C. BARNEBY for identification of this plant.

[22] One-week-old chicks were used for bioassay, with solids being given orally by force feeding in gelatine capsules and liquids or syrups introduced by oral catheter in water solution.

[23] F. A. NORRIS, *Constituents and Toxic Principle of Astragalus Miser*, Utah State University (1970).

and evaporated to yield 0·18 g of a yellow residue shown to be identical to 3-nitro-1-propanol as synthesized below. The aqueous layer was evaporated to yield 0·8 g of an oil which showed a single TLC spot at R_f 0·30 in BAW. A portion of the oil was trimethylsilylated as above and then submitted to GLC analysis at 100° (5ft × 1/8 in. SE-30 column). Peaks were observed only at 80 and 230 sec retention times and the same peaks (corresponding to the two anomers) were observed from trimethylsilylation of a residue from the evaporation of an aqueous solution of glucose.

Miserotoxin was also hydrolysed in D_2O–DCl solution and the NMR of the total hydrolysate was virtually identical to that of a synthetic 1 : 1 mixture of glucose and 3-nitro-1-propanol in D_2O–DCl.

Synthesis of 3-*nitro*-1-*propanol.* Dry silver nitrite (154 g, 1 mole) and 3-bromo-1-propanol (100 g, 0·72 mole) were stirred together in anhydrous ether for 2 days at 25° in the dark. The solution was filtered and the yellow residue washed well with ether. The ethereal solutions were combined, dried, and evaporated to yield an orange oil which was vacuum distilled. The fraction of b.p. 85–87° at 1·4 (lit.[24] b.p. 60–63° at 0·8 mm) was collected to yield 29·9 g (40%) of pure 3-nitro-1-propanol.

Other components. From the butanol extract of the 300 ml aq. crude solution (see above) was isolated a flavonoid (0·4% yield) shown[2] to be 4′-*O*-methylquercetin-3-glucoside. In some early attempts to isolate miserotoxin, the 300 ml aq. crude solution was evaporated to dryness and triturated with MeOH to yield white crystals which could be recrystallized from methanol to a constant m.p. of 184–185°. This was identified as (+)-pinitol (lit.[25] m.p. 186°) by spectral and mixed m.p. comparison with a standard sample.

A. cibarius *Sheld.* Dry, ground plant material (1 kg) was extracted in portions over the course of several days with 5 l. EtOH at 25° by allowing the material to stand in the EtOH, filtering and re-extracting. The combined EtOH extracts were concentrated to 0·5 vol., cooled, and filtered. The ethanolic solution was evaporated to yield about 250 g of an oily dark residue. In a similar preliminary extraction with acetone about 300 g of oily residue was obtained. These two crudes will be referred to as the EtOH crude and the acetone crude.

The EtOH or acetone crude was taken up in a minimum of water and extracted continuously with $CHCl_3$. The $CHCl_3$ solution was evaporated to a thick liquid and silica gel added. The total mixture was then evaporated to dryness. This extract-impregnated silica gel was placed on the top of a dry silica gel column and elutions begun with water-saturated $CHCl_3$. These were followed by elutions with the same solvent to which increments of EtOH had been added. The percent ethanol was critical from 1–10% but less so thereafter and no materials of value were eluted after 50% EtOH : $CHCl_3$. Because of the non-crystallinity of some of the isolated compounds and because some were spread over a considerable number of fractions, yields of each will be quoted as an approximate figure arrived at by a combination of isolated pure compound and NMR analysis of remaining fraction residues. The fraction numbers listed are from an ethanol crude isolation and were approximately the same for compounds isolated from the acetone crude (unless otherwise noted).

Ethyl 3-*nitropropanoate.* From the EtOH crude (but not the acetone crude) an oil was isolated in the first fractions eluted and was identified as ethyl 3-nitropropanoate (0·002%) by comparison of its IR, UV, and NMR spectra with those of a known sample synthesized from EtOH and 3-nitropropanoic acid (Aldrich Chemical Co., Inc.).

3-*Nitropropanoic acid.* From fractions 14–35 (H_2O-saturated $CHCl_3$) was isolated 3-nitropropanoic acid identified by comparison of IR and NMR spectra and m.p. and mixture m.p. with authentic material. The EtOH crude yielded 5 g (0·5%) while the acetone crude yielded 0·2 g (0·02%).

N,N-*Dimethylformamide* (DMF). Fractions 26–29 showed extraneous NMR absorptions. Trituration of the residues with a minimum of anhydrous ether and evaporation of the ether yielded *N*,*N*-dimethylformamide (about 0·001%) identified by IR and NMR comparisons with a known sample. The yield was approximately the same from both the EtOH and acetone crudes.

Each of the solvents used in the above isolation were evaporated in the amounts used and the residue (if any) checked for the presence of DMF, but none was found. The solvents were also used in a complete blank experiment involving evaporation, redissolving and passing through an identical silica gel column, but again no DMF was isolated. A check of the isolation drying, and packing procedure revealed no avenue for the adventitious introduction of DMF.

Succinic and malonic acids. Fractions 36–62 (3% EtOH : $CHCl_3$) yielded a gummy semi-solid residue which was rechromatographed on silica gel. From this rechromatography was isolated 0·05 g (0·05%) of succinic acid and 0·005 g (0·005%) of malonic acid, both identified by m.p. and IR comparison with authentic samples. Yields were approximately the same from the ethanol or acetone cruds.

A di-3-*nitropropanoyl glucose.* Fractions 80–194 from the acetone crude (and about 80–90 from the EtOH crude) all yielded non-crystalline yellow residues having nearly the same NMR spectra. The combined weights of these fractions were 8 g (0·8%) from the acetone crude and 0·05 g (0·05%) from the EtOH crude. Rechromatography of the material in a similar manner to that just described yielded considerable 3-nitropropanoic acid in the early fractions and, in the late fraction, yellow oily residues having virtually the same

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[24] W. F. Baitinger, P. von R. Schleyer, T. S. S. Murty and L. Robinson, *Tetrahedron* **20**, 1635 (1964).

[25] D. C. Pease and R. C. Elderfield, *J. Org. Chem.* **5**, 198 (1940).

NMR as Fractions 87–194 above. Mild aqueous hydrolysis of the oily residue followed by evaporation of the solution, dissolving in EtOH–$CHCl_3$ and rechromatography yielded only 3-nitropropanoic acid. Glucose was identified from the original aqueous acid hydrolysate by chromatographic comparisons with standard samples. Attempted purifications mostly resulted in partial hydrolysis. Combustion analyses yielded values closest to diesters of 3-nitropropanoic acid with glucose (rather than mono- or triesters), but were not exact or consistent. NMR analyses as well as chemical ionization mass spectrometry confirmed this idea and suggested a 1,6-diester structure. Detailed report of these results will be deferred until a complete characterization has been accomplished.

A. curvicarpus *extraction.* In a similar manner to that described above, 50 g of dried plant material was extracted to yield an ethanolic solution. This solution was evaporated to ½ its original volume and allowed to stand for one week at room temp. At the end of this time, white crystals had deposited. These were collected and dried and found to be KNO_3 (m.p. 329–330°) by comparison with an authentic sample. The ethanol solution was then evaporated to dryness.

The EtOH crude was taken up in a minimum of water and extracted with $CHCl_3$. The aqueous layer was evaporated to a gummy residue, which upon addition of methanol, yielded crystalline (+)-pinitol.

The $CHCl_3$ extract was dried and evaporated to a small volume of solution and allowed to stand for one week. A crystalline precipitate was obtained which, upon recrystallization from $CHCl_3$ yielded pure 3-nitropropanoic acid.

Key Word Index—Astragalus; Leguminosae; aliphatic nitro compounds; miserotoxin; 3-nitropropanoic acid.