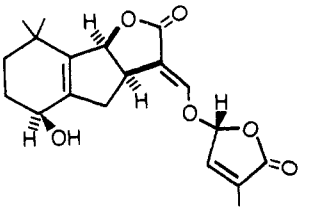
Preparation, Isolation, and High-Performance Liquid Chromatographic Separation of Diastereomers of a Strigol Analogue

William J. Connick, Jr.,* and Armand B. Pepperman, Jr.

A strigol analogue that is known to be an effective witchweed (*Striga asiatica*) seed germination stimulant was prepared by using a new coupling reaction step. The crude product was separated by crystallization procedures into its two diastereomeric racemates, which were isolated and purified for the first time. Spectral and TLC data are reported. High-performance liquid chromatographic (HPLC) separation of the diastereomers was readily accomplished by normal and reverse-phase techniques.

Strigol (1; relative configuration) is a highly effective

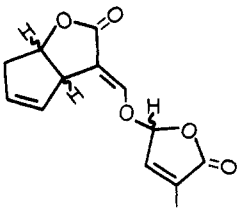


(1)

germination stimulant (Cook et al., 1966, 1972) for seeds of the witchweed plant [*Striga asiatica* (L.) Kuntze (=*Striga lutea*)], a parasite that is a worldwide threat to such important gramineous crops as corn and sorghum. Effective control of witchweed may be possible, especially in tropical countries, if a stimulant other than ethylene was readily available (Johnson et al., 1976).

Reported strigol syntheses (Heather et al., 1976; MacAlpine et al., 1976) are lengthy and uneconomical. As a result, several investigators (Johnson and Rosebery, 1977; Kendall et al., 1979) have sought to prepare simpler, but structurally related, compounds that retain high germination activity.

One such strigol analogue (2), 3-[[(2,5-dihydro-4-



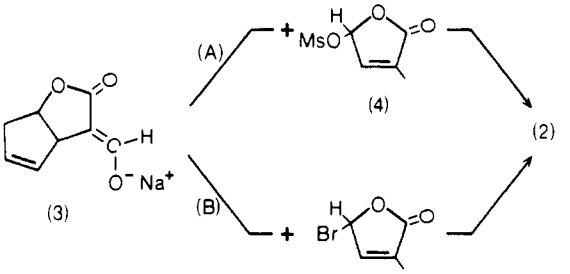
(2)

methyl-5-oxo-2-furanyl)oxy]methylene]-3,3a,6,6a-tetrahydro-2*H*-cyclopenta[*b*]furan-2-one (commonly known as GR-7), was first prepared by Johnson and Rosebery (1977) but not separated into its diastereomers. In 2, the cis 5–5 ring fusion and the chiral center in the butenolide ring give rise to four stereoisomers, two diastereomeric pairs of enantiomers. The ethylenic bridge exclusively has *E* stereochemistry, as determined by ^{1}H NMR (MacAlpine et al., 1976).

Significantly different witchweed seed germination activities have been reported by Heather et al. (1976) for diastereomers of strigol and by Kendall et al. (1979) for diastereomers of an aromatic analogue of strigol. The purpose of the present work was to prepare a quantity of 2 and separate its diastereomers for future evaluation of their seed germination activity. Another objective was to develop a method of separating a mixture of the diastereomers using high-performance liquid chromatography (HPLC).

Southern Regional Research Center, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, New Orleans, Louisiana 70179.

Scheme I. Coupling Reactions



EXPERIMENTAL SECTION

Infrared spectra were obtained on a Perkin-Elmer Model 247 grating spectrophotometer, and ^{1}H NMR on a Varian EM-360L spectrometer (chemical shifts are expressed in ppm downfield from internal tetramethylsilane at $\delta = 0$). Mass spectra were obtained on a Finnigan Series 4000 instrument (solid sample probe) at 70 eV, and ultraviolet spectra on a Hewlett-Packard Model 8450A UV–vis spectrophotometer. Melting points are uncorrected.

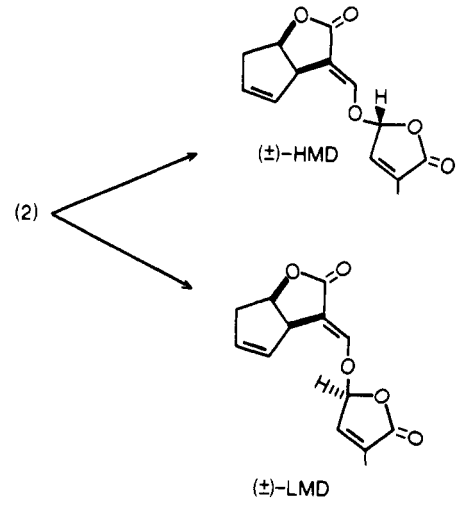
Chromatography. A Waters Associates HPLC was used that consisted of a Model 6000A pump, Model U6K injector, Model 440 UV absorbance detector, and a Model R401 differential refractometer. The detectors were connected to a Model B5217-1 OmniScribe (Houston Instruments) 10-mV strip chart recorder. Normal-phase separations were accomplished with a μPorasil (10 μm), 3.9 mm i.d. × 30 cm analytical column (Waters Associates, PN 27477). Reverse-phase work was done on a Zorbax ODS (5–6 μm), 4.6 mm i.d. × 25 cm analytical column (Du Pont, PN 850952-702).

Hexane and chloroform (with 1% v/v ethanol) were obtained from Burdick and Jackson Laboratories; acetone was Mallinkrodt, Inc., reagent grade; acetonitrile (HPLC grade) was from J. T. Baker Chemical Co. Deionized water was further purified by passage through C_{18} SEP-PAK (Waters Associates, PN 51910) cartridges (one per liter). Prior to use, all solvents were passed through 0.45- or 0.5-μm filters (Millipore Corp.).

Thin-layer chromatography (TLC) was done with 0.25-mm silica gel 60F-254 precoated plates with fluorescent indicator (EM Laboratories, No. 5760). Spots were detected with short-wavelength UV light.

Synthesis. The sodium enolate (3) was prepared according to Johnson and Rosebery (1977), and the bromobutenolide (5) was made as outlined in Heather et al. (1976).

Scheme II. Separation of 2 into Diastereomeric Racemates (Only One Enantiomer of Each Is Shown)[a]



[a] Relative stereochemistry was assigned on the basis of melting points and TLC mobilities as compared with strigol and its epimers (Heather et al., 1976).

Preparation of Strigol Analogue 2 by Scheme I (B). To 2.90 g (16.4 mmol) of bromobutenolide (5) dissolved in 40 mL of acetonitrile was rapidly added 3.13 g (18 mmol, 1.1 equiv) of sodium enolate (3) while stirring and maintaining a dry, inert atmosphere. After being stirred at ambient temperature for 23 h, the mixture was vacuum filtered and the solids were washed with acetonitrile. Evaporation of the filtrate under reduced pressure (35 °C bath) gave 4.04 g of a brown oil. The oil was dissolved in dichloromethane (10 mL), washed with water (2 × 10 mL), and dried over $MgSO_4$. Solvent was removed under reduced pressure (40 °C bath) to give 3.42 g (84%) of 2 as an oil.

Separation of 2 into Diastereomers (Scheme II). *(I) Higher Melting Diastereomers [(±)-HMD].* Crystallization of 3.42 g of 2 from dichloromethane (5 mL)–ether (10 mL) gave 0.75 g of a yellow solid that melted between 152 and 165 °C (with decomposition). The filtrate was saved for part II. Two recrystallizations from dichloromethane (8 mL)–ether (12–20 mL) gave 0.35 g of (±)-HMD as white platelets: mp 174–176 °C (dec); calcd mass for $C_{13}H_{12}O_5$ 248.2; found *m/e* 248; IR (KBr disc) 1785, 1733, 1680, 1351, 854 cm^{-1}; ^1H NMR ($CDCl_3$), all signals are multiplets, δ (centers) 2.00 (3 H), 2.68 (2 H), 4.07 (1 H), 5.05 (1 H), 5.65 (2 H), 6.18 (1 H), 6.95 (1 H), 7.38 (1 H); UV (CH_3CN) λ_{max} 232 nm (ε 20 000); TLC, R_f = 0.48 (chloroform–acetone, 4:1); HPLC (normal phase) showed a single peak.

(II) Lower Melting Diastereomers [(±)-LMD]. The filtrate from part I was evaporated, and the residue crystallized from dichloromethane (2 mL)–ether (30 mL) to give 1.05 g of yellow crystals: mp 121–131 °C (dec). Recrystallization, once from ethanol–water and then 3 times from ether (anhydrous), gave 0.2 g of (±)-LMD as white crystals: mp 143–145 °C (dec); IR (KBr disc) 1783, 1740, 1684, 1335, 877 cm^{-1}; TLC, R_f = 0.53 (chloroform–acetone, 4:1); HPLC (normal phase) showed 97% (±)-LMD–3% (±)-HMD only; mass spectrum, ^1H NMR, and UV results were identical with those of (±)-HMD.

RESULTS AND DISCUSSION

Strigol analogue 2 was prepared according to Johnson and Rosebery (1977), the final step of which is shown in Scheme I (A). However, the unstable mesylate 4 was prepared in higher yield by the method of Crossland and Servis (1970). An alternate coupling reaction [Scheme I (B)] that was used for the first time to prepare 2 is described under Experimental Section. Based on the method of Cassady and Howie (1974), it requires reaction of the sodium enolate (3) with the bromobutenolide (5).

Table I. Melting Points and TLC Mobilities of Witchweed Seed Germination Stimulants

compound	mp, °C	TLC,[a] R_f
(±)-strigol[b]	203–205 (dec)	0.20
(±)-4′-epistrigol[b]	178–180	0.32
(±)-HMD	174–176 (dec)	0.48
(±)-LMD	143–145 (dec)	0.53

[a] Silica gel; chloroform–acetone, 4:1. [b] Heather et al. (1976).

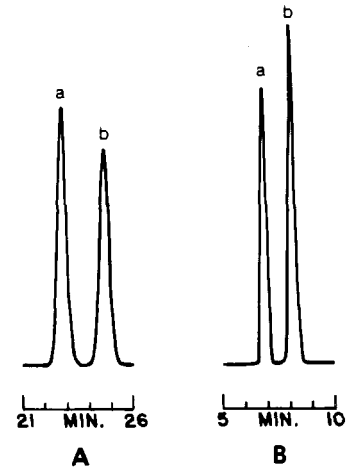


Figure 1. HPLC chromatograms. (A) Reverse phase: Zorbax ODS; acetonitrile (25%)–water (75%); 1.5 mL/min; 1500 psi; UV detector at 254 nm; peak a = (±)-HMD; peak b = (±)-LMD. (B) Normal phase: μPorasil; hexane (60%)–acetone (20%)–chloroform (20%); 1.0 mL/min; 150 psi; RI detector; peak a = (±)-LMD; peak b = (±)-HMD.

Separation of diastereomeric racemates (±)-HMD and (±)-LMD by crystallization gave good purity. The more easily isolated was (±)-HMD. The diastereomers had essentially identical mass, UV, and ^1H NMR spectra. There are some differences in their infrared spectra, notably at 1351 and 854 cm^{-1} for (±)-HMD and 1335 and 877 cm^{-1} for (±)-LMD.

There is a correlation between TLC mobility and melting point for (±)-strigol and (±)-4′-epistrigol and the analogues described here (Table I). It will be determined later if this correlation extends to their relative potency as witchweed seed germination stimulants.

HPLC proved to be an effective technology for separating and analyzing mixtures of these isomers. A mixture of (±)-HMD (52%)–(±)-LMD (47%) was adequately separated (Figure 1A) by reverse-phase HPLC (α = 1.09; R = 2.27) using an analytical C_{18} column with a mobile phase of acetonitrile (25%)–water (75%). A faster analysis (Figure 1B), requiring only ~9 min, was obtained with a normal phase (α = 1.34; R = 2.13) using a silica analytical column with a mobile phase of hexane (60%)–acetone (20%)–chloroform (20%). Note that the isomers elute in opposite order in reverse-phase HPLC compared with normal-phase HPLC. Using a preparative-scale HPLC, it should be possible to separate (±)-HMD and (±)-LMD directly from a crude reaction mixture more efficiently than by crystallization.

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Incorporation of Maleic Hydrazide into Ribonucleic Acid of *Saccharomyces cerevisiae*

Martin D. Appleton,* Walter Haab, Michael L. Eisenstadt, Richard Rodgers, and Charles J. Thoman

Maleic hydrazide [MH; 6-hydroxy-3(2*H*)-pyridazinone] was incubated with *Saccharomyces cerevisiae* and was found to be incorporated into the RNA of the cells where it substituted for cytosine rather than for uracil, its structural isomer. Incorporation was found to take place in the undifferentiated RNA fraction, as well as in tRNA, and may explain the mode of action of MH with regard to its ability to interfere with protein biosynthesis and cell growth.

Maleic hydrazide [MH; 6-hydroxy-3(2*H*)-pyridazinone] has enjoyed extensive use as a commercial plant growth inhibitor since its introduction in 1949 (Schoene and Hoffmann, 1949). Dilution of as low as 1 ppm (Leopold and Klein, 1951) inhibits the growth of a vast multiplicity of plants, but a few derivatives of MH do not exhibit any inhibitory activity at all (Greulach, 1953; Baskokov and Melnikov, 1954; Parups et al., 1962; Greulach and Plylar, 1966).

Kalinin and Troyan (1973) have confirmed and clarified earlier studies by showing that the MH–triethanolamine salt inhibited the incorporation of purine and pyrimidine bases and their nucleotides into nucleic acids, thereby inhibiting RNA and DNA synthesis (Ito and Yoshinaka, 1964; Evans and Scott, 1964; Dubinina and Dubinin, 1967; Kihlman and Hartley, 1967; Lobov, 1971, 1973) and thus interfering with amino acid incorporation into polypeptide chains, causing the accumulation of free amino acids (Biswas et al., 1966) and decreasing the protein content of the cells (Ito and Yoshinaka, 1964; Kato, 1970; Lobov, 1971, 1973).

The pyrimidines thymine, uracil, and thiouracil are antagonists of MH in that they will counteract its inhibiting properties (Butenko and Baskakov, 1961; Povolotskaya, 1961; Coupland and Peel, 1972a,b), suggesting that MH interferes with pyrimidine metabolism (Greulach, 1955). Since MH and uracil are isomers, a logical hypothesis would be that the former acts by interfering with the metabolism of the latter. Such a conclusion was made (Callaghan and Grun, 1961; Callaghan et al., 1962), when MH incorporation was found to follow the pathways of RNA synthesis.

Most of the above work was carried out on intact plants or cells derived from them. The purpose of the research presented here was to incubate MH with the single-celled *Saccharomyces cerevisiae* and to determine its fate within the cell as a means of gaining possible insights into its mode of activity.

Department of Chemistry, University of Scranton, Scranton, Pennsylvania 18510.

EXPERIMENTAL SECTION

In each of the following cases, 15 untreated control samples were run concurrently with 15 samples incubated with MH.

(1) Determination of MH in RNA. (*A*) *Cultivation of Yeast Cells.* Twenty-five cultures of *S. cerevisiae* were prepared in a medium consisting of glucose (40 g), sucrose (60 g), peptone (7 g), KH_2PO_4 (4 g), and $MgSO_4$ (2 g) in 1 L of distilled water (Lindegren, 1962). The pH was adjusted to 4.4 with diluted H_2SO_4, and the mixture subjected to continuous aeration at 36–38 °C for 2 h. Then a solution of MH (0.122 g) in 50 mL of distilled water was added to half the samples (giving solutions 0.001 M in MH) and 50 mL of distilled water was added to the rest, and the cultures were further incubated at 36–38 °C for an additional 2 h.

(*B*) *Isolation of the RNA.* The method is that of Georgiev et al. (1963) as modified by Rubin (1965). The above yeast cells were harvested by centrifugation at 1450*g* for 15 min at 0 °C. They were weighed and placed in a Mason jar with twice their weight of fine glass beads. They were then ruptured by using an Omnimixer at 5000 rpm for 40 min at 0 °C. To this cell homogenate were added 50-mL portions of 0.14 M NaCl and 90% phenol (adjusted to pH 6.0 with 1 N NaOH), as well as EDTA (0.12 g) to inhibit RNase activity. This mixture was stirred at 1000 rpm for 15 min at 0–10 °C and then centrifuged at 1450*g* for 15 min at 0 °C. The uppermost aqueous layer was removed with a pipet, and the RNA precipitated from it by adding twice its volume of 95% ethanol containing 2% potassium acetate. The two lower layers were discarded.

(*C*) *Purificaton of the RNA.* To a suspension of the above RNA in 50 mL of citrate buffer (0.05 M, pH 6.9) in

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