Phytochemical Screening of *Cannabis sativa* L. I: Constituents of an Indian Variant

M. LEONARD MOLE, Jr., and CARLTON E. TURNER[x]

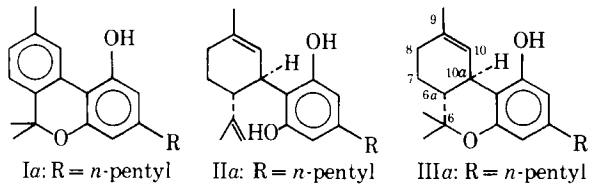
Abstract □ Δ^9-*trans*-Tetrahydrocannabivarin, a mixture of sterols (campesterol, stigmasterol, and β-sitosterol), and the amino acid L-proline were isolated from an Indian variant of *Cannabis sativa* L. Characterizations were accomplished by the usual spectral methods, except for the sterols which were subjected to GLC–mass spectral analysis.

Keyphrases □ Tetrahydrocannabivarin—isolation and characterization from *Cannabis sativa* L. (Indian variant) □ Sterols (campesterol, stigmasterol, and β-sitosterol)—isolation and characterization from *Cannabis sativa* L. (Indian variant) □ L-Proline—isolation and characterization from *Cannabis sativa* L. (Indian variant)

Until the 1940's, the chemistry of *Cannabis sativa* L. remained relatively unexplored and only in the 1960's, with newly accessible modern separation techniques and instrumentation, was major structural work on the cannabinoids accomplished (1). Two groups of workers independently reported confirmation of the structure of the first cannabinoid, cannabinol (I*a*) (2, 3).

However, the structure of cannabidiol (II*a*), the second cannabinoid to be fully characterized, remained obscure until 1963 when the double bond in the terpene moiety was shown (4) to be between carbon atoms 9 and 10 and not between 7 and 8 as previously thought (5, 6).

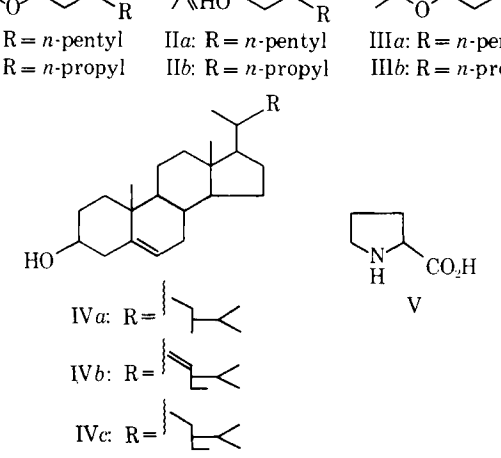
Since that time, about 20 cannabinoids have been isolated and the syntheses of most have been accomplished. Included in this group is the constituent considered to be principally responsible for the psychotomimetic activity of marijuana, (−)-Δ^9-*trans*-tetrahydrocannabinol (III*a*). Also among those isolated are the propyl homologs (*i.e.*, a propyl side chain instead of pentyl) including tetrahydrocannabivarin (III*b*) (7, 8), cannabivarin (I*b*) (8), and cannabidivarin (II*b*) (9).



I*a*: R = *n*-pentyl
I*b*: R = *n*-propyl

II*a*: R = *n*-pentyl
II*b*: R = *n*-propyl

III*a*: R = *n*-pentyl
III*b*: R = *n*-propyl



V

The pharmacological significance of tetrahydrocannabivarin, although only one-fifth as active as III*a* in a catalepsy test on mice (7), has yet to be fully evaluated when taken by humans in the crude state, *e.g.*, smoking marijuana. Gill (7) also reported its effects to be more rapid in onset and decay than III*a* when tested on isolated organs.

In an earlier article from these laboratories (10), significant amounts of tetrahydrocannabivarin and cannabivarin were reported to be present in an Indian variant[1] of *C. sativa* L. It was felt that the isolation of one or both of these constituents would be worthwhile for the following reasons: (*a*) earlier workers had used tinctures or extracts of hashish of generally unknown origin (7–9), (*b*) a supply of III*b* for pharmacological testing could be obtained, and (*c*) confirmation of earlier work might be achieved. The results of these efforts are reported, as well as the isolation of sterols and the amino acid L-proline.

EXPERIMENTAL[2]

Indian plant material, small stems, leaves, and flowering tops which passed through a 14-mesh sieve, was used. Material coded CIX-71, 220 g, and material coded CIF-71, 910 g, were combined and extracted with 95% ethanol for 3 days. The extract was concentrated at aspirator pressure and finally *in vacuo* overnight. The final weight of the dark residue was 106 g. This thick mass was triturated three times with 200-ml portions of hexane. The hexane-soluble portion was concentrated, leaving 55 g of dark gum.

Isolation of Tetrahydrocannabivarin (III*b*)—The cannabinoid-rich gum (hexane soluble), 40 g, was dissolved in 250 ml of hexane, and 175 g of silica gel was added to this solution with swirling. The hexane was evaporated *in vacuo* and the residue was pulverized in a mortar. This somewhat free-flowing material was placed in three equal portions on top of three previously packed columns containing approximately 450 g of silica gel each. The final dimensions were 44 mm × 55 cm. One-liter fractions were taken from each column; combinations were made using GLC analysis. Elution was as follows: fractions 1–15, hexane; 16–

[1] This variant was grown at the University of Mississippi from seed stock produced in the garden. Seeds were obtained originally by Professor Norman J. Doorenbos from Dr. C. K. Atal of the Regional Research Laboratory, Fammutawi, India. Voucher specimens are located in the *C. sativa* L. Herbarium of the School of Pharmacy, University of Mississippi.

[2] GLC analyses were performed using the Beckman GC-45 and GC-72-5 equipped with flame ionization. Glass columns were packed with 2% OV-17 on Chrom "WHP." The inlet temperature was 230°, the column temperature was 210°, and the detector temperature was 250°. Nitrogen was used as the carrier gas at a flow rate of 15 ml/min. IR spectra were taken either on a Beckman IR 33 or a Perkin-Elmer 257 spectrophotometer. UV spectra were taken on a Beckman Acta III. A Dupont 21-492 mass spectrometer was used to obtain mass spectra, and NMR spectra were taken on a Jeol C-60HL. Optical rotations were taken on a Perkin-Elmer 141 polarimeter. Melting points are uncorrected. Column chromatography was performed using Grace grade 923 silica gel, 100–120 mesh, as adsorbent (Davison Chemical, Baltimore, Md.).

18, hexane–benzene (9:1); 19–22, hexane–benzene (4:1); 23–33, hexane–benzene (3:2); 34–44, benzene; 45–49, benzene–chloroform (9:1); 50–53, benzene–chloroform (4:1); 54–56, benzene–chloroform (3:2); 57–59, chloroform; and 60–63, chloroform–methanol (9:1). Solvents were recycled except where solvent system changes were made that would have led to an unwanted system [such as in changing from hexane–benzene (3:2) to benzene].

TLC and GLC analyses of fractions 33 and 34 (2.20 g) indicated that they consisted of one major component (III*b*) and two minor components (I*b* and II*b*). Further purification was accomplished on a column of silica gel impregnated with silver nitrate (20% of total weight). A column, 25 mm × 30 cm, was prepared with 90 g of the adsorbent. Elution collecting 200-ml fractions was as follows: fractions 1–7, benzene; 8–9, ether; and 10, ether–methanol (9:1). Fraction 1, 1.38 g containing 78% III*b* by GLC analysis, was subjected to another chromatography on 59 g of 20% silver nitrate-impregnated silica gel. The column measured 25 mm × 18 cm and elution, taking 150-ml cuts, was as follows: fractions 1–3, hexane; 4–6, hexane–benzene (99:1); 7–9, hexane–benzene (95:5); 10–12, hexane–benzene (9:1); 13–18, hexane–benzene (3:1); 19–21, hexane–benzene (1:1); 22–26, benzene; and 27–28, ether.

Fraction 22, after evaporation of solvent, yielded 250 mg of a colorless oil which darkened rapidly. It was shown by GLC analysis to contain 94% III*b* (relative retention time of 0.28 with 4-androstene-3,17-dione as internal standard). NMR ($CDCl_3$): δ 0.92 (t, J = 7 Hz, aliphatic CH_3), 1.12 and 1.47 (gem-CH_3's), 1.75 (olefinic CH_3), 2.48 (t, J = 8 Hz, C-1′ H's), 3.30 brd, J = 10 Hz, C-10H), 5.56 (s, OH), 6.30 (d, J = 2 Hz, aromatic H), 6.46 (d, J = 2 Hz, aromatic H), and 6.53 (m, C-10H); mass spectroscopy (70 ev, inlet temperature 185°): m/e M^+ 286 (100%), 271 (74), 258 (6), 243 (53), 218 (7.5), 203 (48), and 165 (18), and m^* 257, 206.8, and 189.1; IR (sodium chloride plates): 3410, 2945, 1635, 1590, 1520, 1435, 1190, 1040, 910, and 730 cm^{-1}; $[\alpha]_D^{24}$ −143° (c 1.08 in carbon tetrachloride): [lit. (7) $[\alpha]_D^{20}$ −128° (c 1.0 in carbon tetrachloride)]; λ_{max} (methanol): 279 nm (log ϵ 3.51).

Isolation of a Mixture of Sterols [Campesterol (IV*a*), Stigmasterol (IV*b*), and Sitosterol (IV*c*)]—Fractions 49 and 50 of the first chromatography described were combined (420 mg) and recrystallized three times from ethanol, yielding white crystals (50 mg), mp 134–135°. The NMR spectrum was typical of sterols. TLC showed only one spot, the R_f value of which corresponded to that of cholesterol[3]. NMR ($CDCl_3$): δ 0.70–1.02 (CH_3's) and 5.43 (olefinic H's); IR: 3430, 3320, 3020, 2930, 1640, and 1463 cm^{-1}; GLC–mass spectral data[4]: peak 1 (campesterol), M^+ 400 (31), 382 (91), 367 (32), 281 (11), 207 (34), and 43 (100); peak 2 (stigmasterol), M^+ 412 (20), 394 (52), 351 (17), 255 (61), 133 (42), and 55 (100); peak 3 (β-sitosterol), M^+ 414 (28), 406 (49), 381 (19), 255 (25), and 43 (100).

Isolation of L-Proline (V)—The hexane-insoluble portion, 51 g, of the plant extract was dissolved in chloroform and filtered. The filtrate was extracted with 5% HCl, and the acid extract was made basic with concentrated ammonium hydroxide. This basic solution was concentrated to a paste under vacuum, which was dissolved in 100 ml of methanol and filtered. To the filtrate was added 100 ml of chloroform, and the precipitate was filtered. Concentration of the filtrate left 16.7 g of brown crystalline material, which was dissolved in 20 ml of methanol and chromatographed on 500 g of silica gel (44 mm × 55 cm). Elution was as follows: fractions 1–4, approximately 400 ml of methanol each, and 5–6, approximately 700 ml of ethanol–ammonium hydroxide (9:1) each.

Fraction 5, 125 mg, slowly crystallized; however, an attempt to recrystallize this fraction from methanol–chloroform resulted in an oil separating out. The supernate was decanted and allowed to evaporate slowly, leaving a mixture of oil and white crystals. The crystals were quite soluble in chloroform whereas the oil was not; therefore, separation was achieved. Evaporating the chloroform left 70 mg of an oil which was now insoluble in chloroform. This oil was partitioned between chloroform and water. Evaporation of water left 67 mg of crystalline solid. Three recrystallizations from ethanol–acetone afforded 18 mg of slightly colored needles, mp 220° (instant change), 233–234° dec. (melt) (apparatus preheated to 220°), no depression on admixture with authentic L-proline (mp 233–235°); IR: 3415, 2975, 1616, 1560, and 1375 cm^{-2}; NMR (D_2O): δ 4.73 (t, J = 7.5 Hz, 1H, N—$CHCO_2$), 3.93 (t, J = 6 Hz, 2H, N—CH_2), and 2.62 (m, 4H, CH_2CH_2); $[\alpha]_D^{24.5}$ −71.1° (c 0.95 in water) [lit. (11) $[\alpha]_D$ −85.0 (c 1.0)]; mass spectroscopy (70 ev, inlet temperature 252°): m/e M^+ 115 (3), 87 (2), 70 (100), 43 (19), and 41 (14)[5].

RESULTS AND DISCUSSION

(−)-Δ^9-*trans*-Tetrahydrocannabivarin (III*b*) was isolated from an Indian variant of *C. sativa* L. and purified by extensive column chromatography. To the knowledge of the authors, this represents the first isolation of this cannabinoid from plant material of confirmed origin. Physical data agree well with those published (7, 8). The mass spectrum shows fragmentations that are expected by analogy with those reported for the pentyl homolog (12–14). These fragmentations were discussed in detail previously (13, 14). In addition, the NMR spectrum agreed well with published data, the outstanding feature being the highly deshielded vinyl proton (C-10), δ 6.54. This is quite characteristic of the 9,10-double bond, as discussed previously (15) in reference to III*a*.

There were, however, some minor discrepancies in the IR spectrum when compared with that previously observed (7). It was reported that the IR spectra of III*a* and III*b* were essentially the same, except for the presence of a band at 2950 cm^{-1} in III*a* and some difference in the relative intensities of the two portions of the doublet at 1038 and 1048 cm^{-1}. By contrast, in the present study the spectra of the two compared well except for a more intense band for III*a* at 2950 cm^{-1} and the presence of two bands for III*b* of medium intensity at 910 and 730 cm^{-1}. Perhaps these discrepancies can be attributed to the different phases used when obtaining the spectra.

Additional column chromatography after III*b* was isolated afforded fractions rich in sterols. Constant melting material was obtained by recrystallization; however, GLC analysis indicated a mixture. Further analysis by GLC–mass spectrometry revealed three components whose mass spectra matched well with those corresponding to campesterol (IV*a*), stigmasterol (IV*b*), and β-sitosterol (IV*c*). The latter two are probably the most commonly occurring sterols in higher plants. To the authors' knowledge, this is the first report of sterols having been isolated from *C. sativa* L. of known origin grown under controlled conditions. Fenselau and Hermann (16) reported isolation of a mixture of these three sterols, which were characterized by GLC–mass spectroscopy, from red oil extract of Yugoslavian *Cannabis*.

Column chromatography of the polar portion of the extract yielded L-proline (V), which was purified in the somewhat unorthodox manner discussed previously. Assignment of structure was based on the mass spectrum which showed the base peak, m/e 70 (M − 45), characteristic of L-proline (17, 18). Likewise, the IR and NMR spectra matched those of an authentic sample. Even though this commonly occurring amino acid would be expected to be found in *C. sativa* L., this is the first report of its having been isolated[6].

SUMMARY

Chemical screening of an Indian variant of *C. sativa* L. resulted in the isolation of Δ^9-*trans*-tetrahydrocannabivarin, three sterols (campesterol, stigmasterol, and β-sitosterol), and the amino acid L-proline. To the authors' knowledge, this is the first report of these compounds having been isolated from marijuana plant material of known origin.

REFERENCES

(1) J. L. Neumeyer and R. A. Shagoury, *J. Pharm. Sci.*, **60**, 1433(1971).

(2) R. Adams, B. R. Baker, and R. B. Wearn, *J. Amer. Chem.*

[3] Silica gel with two solvent systems: ethyl acetate and chloroform–benzene (3:2).

[4] The authors thank Dr. Paul Schiff, Department of Pharmacognosy, School of Pharmacy, University of Pittsburgh, for the GLC–mass spectroscopic analysis and his helpful comments.

[5] The authors thank Dr. Roger Foltz and Dr. Al Fentiman, Battelle Memorial Institute, Columbus, Ohio, for a mass spectrum and suggestions.

[6] Lousberg and Salemink (19) reported the composition of hydrolyzed amino acids in *C. sativa* L. determined with an amino acid analyzer.

Soc., 62, 2204(1940).
(3) R. Ghosh, A. R. Todd, and S. Wilkinson, *J. Chem. Soc.*, 1940, 1121.
(4) R. Mechoulam and Y. Shvo, *Tetrahedron*, 19, 2073(1963).
(5) R. Adams, S. Loewe, D. C. Pease, D. K. Cain, R. B. Wearn, B. R. Baker, and H. Wolff, *J. Amer. Chem. Soc.*, 62, 2566(1940).
(6) R. Adams, C. K. Cain, W. D. McPhee, and R. B. Wearn, *ibid.*, 63, 2209(1941).
(7) E. W. Gill, *J. Chem. Soc.*, 1971, 579.
(8) F. W. H. M. Merkus, *Nature*, 232, 579(1971).
(9) L. Vollner, D. Bieniek, and F. Korte, *Tetrahedron Lett.*, 1969, 145.
(10) P. S. Fetterman and C. E. Turner, *J. Pharm. Sci.*, 61, 1476(1972).
(11) "The Merck Index," 8th ed., Merck & Co., Rahway, N.J., 1968, p. 869.
(12) H. N. Fales, G. W. A. Milne, and N. C. Law, *Arch. Mass Spectral Data*, 2, 652(1971).
(13) U. Claussen, H. W. Fehlhaber, and F. Korte, *Tetrahedron*, 22, 3535(1966).
(14) H. Budzikiewicz, R. T. Alpin, D. A. Lightner, C. Djerassi, R. Mechoulam, and Y. Gaoni, *ibid.*, 21, 1881(1965).
(15) Y. Gaoni and R. Mechoulam, *J. Amer. Chem. Soc.*, 86, 1646(1964).
(16) C. Fenselau and G. Hermann, *J. Forensic Sci.*, 17, 309(1972).
(17) G. Junk and H. Svec, *J. Amer. Chem. Soc.*, 85, 839(1963).
(18) K. Heyns and H. F. Grutzmacher, *Ann.*, 667, 194(1963).
(19) R. J. J. Ch. Lousberg and C. A. Salemink, *Pharm. Weekbl.* 108, 1(1973).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 22, 1973, from the *Research Institute of Pharmaceutical Sciences and the Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, MS 38677*

Accepted for publication September 14, 1973.

Presented in part to the American Society of Pharmacognosy and to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Jekyll Island, Ga., meeting, July 1973.

Supported by Contract HSM-42-70-109 from the National Institute of Mental Health and by the Research Institute of Pharmaceutical Sciences.

[x]To whom inquiries should be directed.

Sulfide, Disulfide, and Sulfone Derivatives of 2-Phenylcinchoninic Acid as Antimalarial Congeners

CHARLES I. SMITH[x] and PILAN LIN HSU

Abstract □ The diethyl ester of bis[6-(2-phenylcinchoninic acid)] sulfone, the corresponding sulfide and disulfide derivatives, and the disulfide acid were prepared. The results of chemotherapeutic testing for antimalarial activity are reported.

Keyphrases □ Bis[6-(2-phenylcinchoninic acid)] sulfone diethyl ester and sulfide and disulfide analogs—synthesized and screened as potential antimalarial agents □ Antimalarial agents, potential —synthesis and screening of bis[6-(2-phenylcinchoninic acid)] sulfone diethyl ester and sulfide and disulfide analogs □ Sulfones, aryl—synthesis and screening of bis[6-(2-phenylcinchoninic acid)] sulfone diethyl ester as a potential antimalarial agent

Elslager *et al.* (1) reported the relationship between chemical structure and antimalarial activity in a series of compounds related to 4,4′-sulfonyldianiline (I), also known as DDS and dapsone. Other investigators reported the synthesis and testing of a variety of sulfones with varying degrees of activity as antimalarial agents (2, 3).

As part of an effort directed toward the synthesis of potential antimalarial agents, it was decided to prepare bis[6-(2-phenylcinchoninic acid)] sulfone diethyl ester (II) as an analog of dapsone. Several structurally related compounds, bis[6-(2-phenylcinchoninic acid)] sulfide diethyl ester (III), bis[6-(2-phenylcinchoninic acid)] disulfide (IV), and bis[6-(2-phenylcinchoninic acid)] disulfide diethyl ester (V), were also prepared for possible structure–activity comparisons.

The synthetic approach to III and IV started with the condensation of the appropriately substituted amines, 4,4′-thiodianiline and *p*-aminophenyl disulfide, respectively, with benzaldehyde and pyruvic acid *via* the Doebner reaction (4). The intermediate acids were converted to the esters, III and V, by a standard procedure (5) for the preparation of aromatic esters. The sulfone was prepared by dichromate oxidation (6) of the corresponding sulfide.

Chemotherapeutic screening of these substances as potential antimalarial agents disclosed no significant antimalarial activity and no detectable host toxicity under the test conditions.

EXPERIMENTAL[1]

4,4′-Thiodianiline[2] and *p*-aminophenyl disulfide, prepared according to the method of Price and Stacy (7), were used.

Bis[6-(2-phenylcinchoninic Acid)] Sulfide Diethyl Ester (III) —To a stirred solution of benzaldehyde (21.2 g, 0.200 mole) in warm ethanol (80 ml), 4,4′-thiodianiline (21.6 g, 0.100 mole) was added rapidly and the resulting mixture was heated at reflux for 10 min. A solution of redistilled pyruvic acid (17.6 g, 0.200 mole) in ethanol (18 ml) was added dropwise over 30 min. The resulting mixture was refluxed and stirred continuously for an additional 6 hr, cooled to room temperature, and stirred overnight. After

[1] Melting points were determined on a Fisher-Johns or Thomas-Hoover melting-point apparatus and are uncorrected. IR spectra were recorded with a Beckman IR-8 spectrophotometer. NMR spectra were recorded with a Varian Associates A-60 spectrometer. Microanalyses were performed by Micro-Analysis, Inc., Wilmington, Del.

[2] Eastman Organic Chemicals, Rochester, N.Y.