The Isolation and Structure of Δ^1-Tetrahydrocannabinol and Other Neutral Cannabinoids from Hashish

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Abstract: The isolation and elucidation of the structures of Δ^1-tetrahydrocannabinol (Δ^1-THC), cannabigerol, cannabichromene, and cannabicyclol are described. A facile conversion of cannabidiol into Δ^1-THC takes place on treatment with boron trifluoride etherate. The absolute configuration of the chiral centers at C-3 and C-4 of Δ^1-THC is established as *R*.

The resin of the female *Cannabis sativa* L. plant has been used as a medicine and a psychotomimetic drug since ancient times.[1] *Cannabis* preparations were known to the Assyrians, Scythians, ancient Chinese, Indians, and Persians. More recently, increased consumption of either the resin (hashish) or the whole flowering top (marihuana) has caused worldwide social, legal, and medical problems.

The chemistry of the constituents of *Cannabis* has been the subject of numerous publications since the middle of the last century.[2] Due mainly to the masterly investigations of Cahn,[3] Adams,[4] Bergel,[5] and Todd[6] substantial progress was made in this field. However, until 1963, when the structure of cannabidiol (Ia) was elucidated,[7] the only cannabinoid with fully known constitution was the inactive cannabinol (II); the *active* constituent had not been isolated in pure form, its structure was not fully known, and it was unavailable from either a natural or a synthetic source. The reason for the slow progress is to be found in the lack of suitable separative and analytical techniques in the thirties and early forties, when the important work in Urbana[4] and Cambridge[6] took place. As reproducible pharmacological and clinical investigations can only be undertaken with well-defined materials, this incomplete chemical evaluation of marihuana resulted in an almost total absence of fundamental experimental work on the biological aspects of the *Cannabis* problem. This lack of data on the pharmacological effects has had, in turn, serious social repercussions in the present wave of marihuana use.

In a number of communications[8] we reported the isolation, structure elucidation, and absolute configuration of some neutral cannabinoids, including the major active constituent, Δ^1-tetrahydrocannabinol (Δ^1-THC). We wish to describe now the full details of this research.

Previous work[2] indicated that the active constituent(s) were found in the petroleum ether extract of hashish. We were able to confirm and extend this observation. Benzene and methanol extracts from hashish, which had previously been repeatedly extracted with petroleum ether, were found to be inactive when tested in rhesus monkeys.[9] Hence we concentrated on the petroleum ether fraction, which was separated into neutral and acidic components. The acid fraction was inactive.[9] The following compounds and mixtures were isolated from the active, *neutral* fraction by repeated chromatography on Florisil or acid-washed alumina, and alumina containing 12% silver nitrate (in order of increasing polarity): (1) a mixture of waxy, noncannabinoid materials; (2) cannabicyclol (III); (3) cannabidiol (Ia);[7] (4) Δ^1-THC (IVa); (5) cannabinol (II); (6) cannabichromene (Va); (7) cannabigerol (VI); and (8) polar constituents and polymers. The yields of cannabinoids are indicated in Table I.

Table I. Content in Hashish,[a] R_f Values (tlc),[b] and Retention Times (vpc)[c] of Some Natural Neutral Cannabinoids

	Yields[a]	R_f[b]	Retention time[c]
Cannabicyclol (III)	0.11	0.62	4′ 33″
Cannabidiol (Ia)	3.74 (1.4) (2.5)	0.58	5′ 40″
$\Delta^{1(6)}$-THC (VIII)	Not detected	0.57	7′ 10″
Δ^1-THC (IVa)	3.30 (1.4) (3.4)	0.51	7′ 52″
Cannabinol (II)	1.30 (0.3) (1.2)	0.47	10′ 12″
Cannabichromene (Va)	0.19	0.43	5′ 35″
Cannabigerol (VII)	0.30	0.42	9′ 20″

[a] As per cent of hashish; determined by vpc. The numbers in parentheses are from two partial analyses of different batches. [b] Chromatoplates of silica gel. Elution with petroleum ether (bp 40–60°) and ether in a ratio of 4:1. [c] Column 2% OV-17 on Gas–Chromosorb Q; N_2 flow, 30 cc/min; column temperature, 235°.

The active Δ^1-THC represented 3.3% of the sample. Partial analyses of different batches showed the presence of 1–5% Δ^1-THC. *Cannabis* preparations vary widely in their content of cannabinoids.[10,11] This

(1) R. J. Bouquet [*Bull. Narcotics*, 3 (3), 22 (1951), and references cited therein] describes in fascinating detail the history of *Cannabis* use and the various preparations and modes of consumption in different parts of the world.

(2) Reviews: R. Mechoulam and Y. Gaoni, *Fortschr. Chem. Org. Naturst.*, **25**, 174 (1967); R. Mechoulam, *Science*, **168**, 1159 (1970).

(3) R. S. Cahn, *J. Chem. Soc.*, 1400 (1933).

(4) R. Adams, *Harvey Lect.*, **37**, 168 (1942).

(5) F. Bergel and K. Vögele, *Justus Liebigs Ann. Chem.*, **493**, 250 (1932).

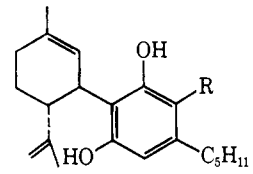
(6) A. R. Todd, *Experientia*, **2**, 55 (1946).

(7) R. Mechoulam and Y. Shvo, *Tetrahedron*, **19**, 2073 (1963).

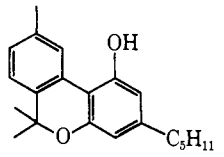
(8) (a) Y. Gaoni and R. Mechoulam, *Proc. Chem. Soc.*, 82 (1964); (b) Y. Gaoni and R. Mechoulam, *J. Amer. Chem. Soc.*, **86**, 1646 (1964); (c) Y. Gaoni and R. Mechoulam, *Chem. Commun.*, 20 (1966); (d) R. Mechoulam and Y. Gaoni, *Tetrahedron Lett.*, 1109 (1967).

(9) (a) R. Mechoulam, A. Shani, H. Edery, and Y. Grunfeld, *Science*, **169**, 611 (1970). (b) The monkey tests were performed according to Y. Grunfeld and H. Edery, *Psychopharmacologia*, **14**, 200 (1969).

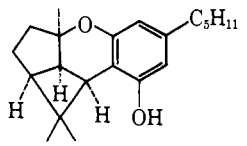
variability may be the result of many factors such as climate, soil, mode of preparation, length of storage, etc. In this connection it should be pointed out that on heating the inactive cannabinoid, acids are decarboxylated to yield the corresponding neutral cannabinoids.[12] Hence for practical estimation of the Δ^1-THC available *on smoking*, the amount of Δ^1-THC acid A (IVb)[12c] and Δ^1-THC acid B (IVc)[12d] should be taken into consideration. In hashish these acids constitute 1–3%. The above figures are compatible with the popularly accepted notion that Middle Eastern hashish and Indian charas contain 5–6 times more active material than American marihuana.[11]



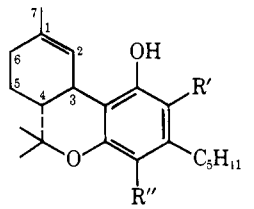
Ia, R = H
b, R = COOH



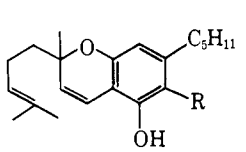
II



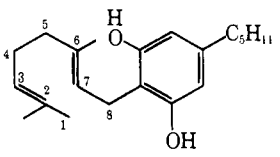
III



IVa, R′ = R″ = H
b, R′ = COOH; R″ = H
c, R′ = H; R″ = COOH



Va, R = H
b, R = COOH



VI

Δ^1-THC (IVa). The only active[9] cannabinoid isolated by us was Δ^1-THC. If other active constituents were present in the neutral, petroleum ether soluble fraction, they were probably very minor components.

The oily Δ^1-THC was further purified *via* the crystalline 3,5-dinitrophenylurethane VIIa followed by hydrolysis. This represents, to the best of our knowledge, the first authenticated case of isolation in a pure form of an active *Cannabis* principle. The 3,5-dinitrophenylurethane VIIa is the only *crystalline* derivative of natural Δ^1-THC known so far. The urethane, which was obtained by boiling Δ^1-THC with 3,5-dinitrophenyl azide, was accompanied by the amide VIIb. The formation by an isocyanate of an aromatic amide in the presence of a free phenolic group is very unusual.

The structure of Δ^1-THC (IVa) was established by spectroscopic measurements and chemical correlations. Dehydrogenation of Δ^1-THC gave cannabinol (II) in almost quantitative yield. Boiling Δ^1-THC with *p*-toluenesulfonic acid converted it into $\Delta^{1(6)}$-THC (VIII).[13–15] The spectroscopic data fit the suggested structure (see Experimental Section). The uv spectrum indicates that the double bond is not conjugated with the aromatic ring. The nmr spectrum shows the presence of only one aliphatic methyl group and of three methyl groups which are either α to an oxygen or are vinylic. This observation places the double bond in the Δ^1 or $\Delta^{1(6)}$ position. It is of interest to compare the chemical shifts of the C-2 and C-3 protons in Δ^1-THC (IVa) and in cannabidiol (Ia).[7] The olefinic proton in IVa (δ 6.35) is deshielded as compared to that in Ia (δ 5.59), while the reverse relationship exists as regards the C-3 protons (IVa, δ 3.14; Ia, δ 3.85). This can be readily understood by examination of molecular models of these two compounds. In cannabidiol, the aromatic ring, which can rotate freely, is most probably in the same plane as the C-3 hydrogen, which is therefore deshielded. In Δ^1-THC the additional ring tilts the aromatic ring, so that the latter is now in (or nearly in) the same plane as the olefinic proton, which is therefore deshielded. Such an effect is possible only if the double bond occupies the Δ^1 position and the protons of the two chiral carbons are trans, *i.e.*, THC possesses structure IVa. When reported in 1964[8b] this represented the first structure elucidation of an active *Cannabis* component.

This structure is supported by a partial synthesis. Reaction of cannabidiol (Ia) with boron trifluoride etherate in methylene chloride gave a mixture, from which 60% Δ^1-THC and 13% Δ^8-*i*-THC (IX)[13,16] were isolated. Alternatively cannabidiol can be converted quantitatively into $\Delta^{1(6)}$-THC (VIII)[13,14] and then into Δ^1-THC by hydrochlorination and dehydrochlorination.[17] In view of the ready availability of cannabidiol from cannabidiolic acid (Ib)[12a,b] in hemp these partial syntheses should make pure Δ^1-THC readily available for psychobiological experimentation.

Numerous total syntheses of racemic as well as (−)- and (+)-Δ^1-THC have been reported.[17,18]

For many years a mixture of unidentified tetrahydrocannabinol isomers was assumed to be responsible for the activity of *Cannabis*.[6] This belief stemmed mainly from the synthetic work of Adams[4] and Todd[6] who prepared X as an intermediate in the synthesis of cannabinol. This unnatural THC (X) was active in the dog ataxia and rabbit blinking tests, which were assumed to parallel the activity in humans. In another investigation[15] Adams found that acid isomerizations of cannabidiol, which at that time was considered to possess structure XI, gave mixtures of active tetrahydrocannabinols, which were not identical with X.[19]

(10) *Cf.* T. W. M. Davis, C. G. Farmilo, and M. Osadchuk, *Anal. Chem.*, **35**, 751 (1963).

(11) M. Lerner and J. T. Zeffert, *Bull. Narcotics*, 20 (2), 53 (1968).

(12) (a) J. Kabelic, Z. Krejči, and F. Šantavý, *ibid.*, 12 (3), 5 (1960); (b) R. Mechoulam and Y. Gaoni, *Tetrahedron*, **21**, 1223 (1965); (c) U. Claussen and F. Korte, *Justus Liebigs Ann. Chem.*, **713**, 162 (1968); (d) R. Mechoulam, Z. Ben-Zvi, B. Yagnitinsky, and A. Shani, *Tetrahedron Lett.*, 2339 (1969).

(13) Y. Gaoni and R. Mechoulam, *Tetrahedron*, **22**, 1481 (1966).

(14) (a) R. L. Hively, W. A. Mosher, and F. W. Hoffmann, *J. Amer. Chem. Soc.*, **88**, 1832 (1966); (b) E. C. Taylor, K. Lenard, and Y. Shvo, *ibid.*, **88**, 367 (1966).

(15) R. Adams, C. K. Cain, W. D. McPhee, and R. B. Wearn, *ibid.*, **63**, 2209 (1941), and papers cited therein.

(16) Y. Gaoni and R. Mechoulam, *Israel J. Chem.*, **6**, 679 (1968).

(17) (a) R. Mechoulam, P. Braun, and Y. Gaoni, *J. Amer. Chem. Soc.*, **89**, 4552 (1967); (b) K. E. Fahrenholtz, M. Lurie, and R.W. Kierstead, *ibid.*, **89**, 5934 (1967); (c) T. Petrzilka, W. Haefliger, and C. Sikemeier, *Helv. Chim. Acta*, **52**, 1102 (1969).

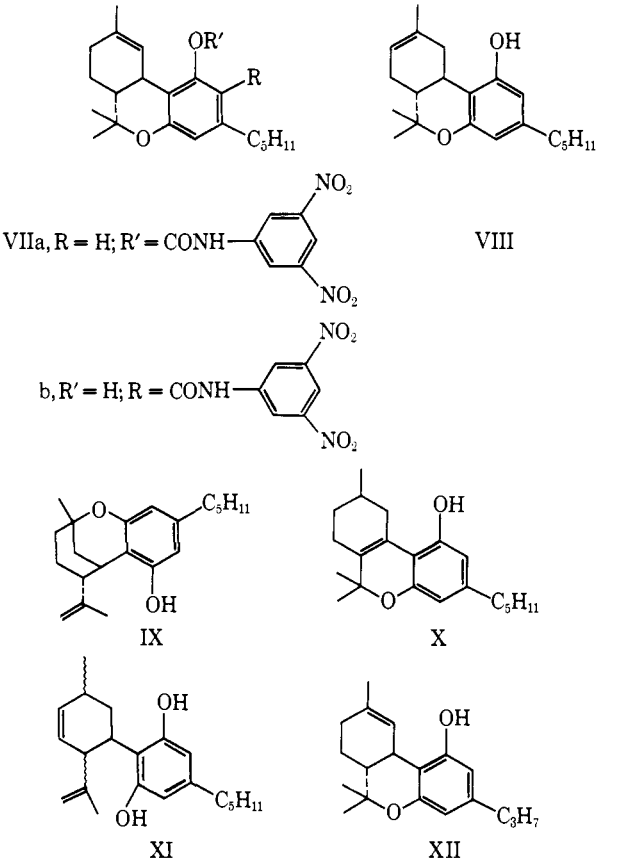
(18) (a) R. Mechoulam and Y. Gaoni, *J. Amer. Chem. Soc.*, **87**, 3273 (1965); (b) T. Y. Jen, G. A. Hughes, and H. Smith, *ibid.*, **89**, 4551 (1967).

(19) We have reinvestigated[13] these biologically active mixtures and have found that although they contain $\Delta^{1(6)}$-THC (VII) and some Δ^1-THC (IVa), numerous other components are present as well. Boiling

In 1942 Wollner, *et al.*,[20] isolated from marihuana an active product which could be converted into cannabinol (II). It was different from the synthetic and semisynthetic THC's prepared by Adams. No definite structure was proposed for this material, though it was assumed to be a THC isomer. The $[\alpha]D$ $-193°$ reported for this substance indicates now that it was probably impure Δ^1-THC ($[\alpha]D$ $-150°$). A few additional reports on the isolation of active materials from *Cannabis* have appeared in the literature. Haagen-Smit, *et al.*,[21] and Powell, *et al.*,[22] have published short communications on the isolation of active materials. These reports lack details to allow comparison with later work. de Ropp[23] has described the isolation of a THC. Its infrared spectrum and some other physical properties are similar to those of Δ^1-THC. These reports deal mainly with the isolation of material and do not contribute any additional data as to the structure(s).

The fact that different THC's or mixtures of isomers showed activity led to the generally accepted belief that the activity of *Cannabis* was due to a mixture of isomers. This view is in our opinion not correct, the activity being due largely, or almost exclusively, to Δ^1-THC. However two additional active compounds have been identified. Hively, *et al.*,[14a] reported the presence of $\Delta^{1(6)}$-THC (VIII) in marihuana: the ratio of $\Delta^{1(6)}$-THC to Δ^1-THC was *ca.* 1:10. Most *Cannabis* samples, which have been analyzed, however, contain considerably less $\Delta^{1(6)}$-THC, the ratio being *ca.* 1:100 or even lower.[11] The Δ^1 isomer is more labile than the $\Delta^{1(6)}$ isomer; hence the ratio will vary also depending on the length and conditions of storage. Very recently an active homolog of Δ^1-THC was identified in Pakistani hashish. It has been assigned[24] structure XII, and is 4.8 times less active than Δ^1-THC in its cateleptic activity in mice. This homolog does not seem to to be present in hashish on the basis of vpc measurements.

In our preliminary communication[8b] we reported that Δ^1-THC showed strong ataxia activity in dogs. Full details of the animal tests have since been published.[9b] *Detailed* human experiments were not undertaken by us, but on the basis of preliminary tests on volunteers we reported that the effective dose in humans was 3–5 mg.[2] Later, numerous groups reported on the activity of Δ^1-THC in animals and humans.[25] However as yet our understanding of the molecular basis of THC action is negligible.



Cannabigerol (VI). This minor component is the only known neutral cannabinoid whose stage of oxidation is lower than that of the rest of the group. We have assumed[8a] that cannabigerol is formed in nature from geraniol and olivetol and hence represents the initial product of cannabinoid biogenesis.

Cannabigerol has two reducible double bonds (as determined by microhydrogenation). The uv spectrum of cannabigerol shows the absence of conjugation. The nmr spectrum indicates that (a) the two aromatic hydrogen atoms are magnetically equivalent, (b) the protons of the methylene group at C-8 are strongly deshielded, and split by a single adjacent proton, which is presumably due to the Δ^6 double bond, and (c) three olefinic methyl groups are present, which suggests that the second double bond is at the Δ^2 position. Assuming that the side chain is of the normal terpenoid type these findings are compatible with structure VI only.

The structure of cannabigerol has been confirmed by syntheses.[8a,26]

Cannabichromene (Va). This minor component was isolated independently by Claussen, *et al.*,[27] and by us[8c] and through a coincidence was given the same name by both groups. The $[\alpha]D$ was reported as $+3.4$ and $-9°$. Later work[16] has indicated that when the oily cannabichromene is further purified, no rotation is observed. A crystalline derivative, a 3,5-dinitrophenylurethane, mp 106–107°, has likewise no rotation. Cannabichromene (Va) has been correlated with cannabidiol (Ia) *via* $\Delta^{4(8)}$-*i*-THC (XIII).[16] This compound when obtained from cannabidiol has a rotation of $[\alpha]D$ $-300°$, while when prepared from

cannabidiol (Ia) with *p*-toluenesulfonic acid, however, gives essentially one product, $\Delta^{1(6)}$-THC, to which Adams, *et al.*, correctly assigned structure VIII (without stereochemistry).[15] Δ^1-THC (IVa) was not isolated in pure form either from a natural material or from a semisynthetic mixture by the groups in Urbana[4] or Cambridge.[6]

(20) H. J. Wollner, J. R. Matchett, J. Levine, and S. Loewe, *J. Amer. Chem. Soc.*, **64**, 26 (1942).

(21) A. J. Haagen-Smit, C. W. Wawra, J. B. Koepfli, G. A. Alles, G. A. Feigen, and A. N. Prater, *Science*, **91**, 602 (1940).

(22) G. Powell, M. R. Salmon, T. H. Bembry, and R. P. Walton, *ibid.*, **93**, 522 (1941).

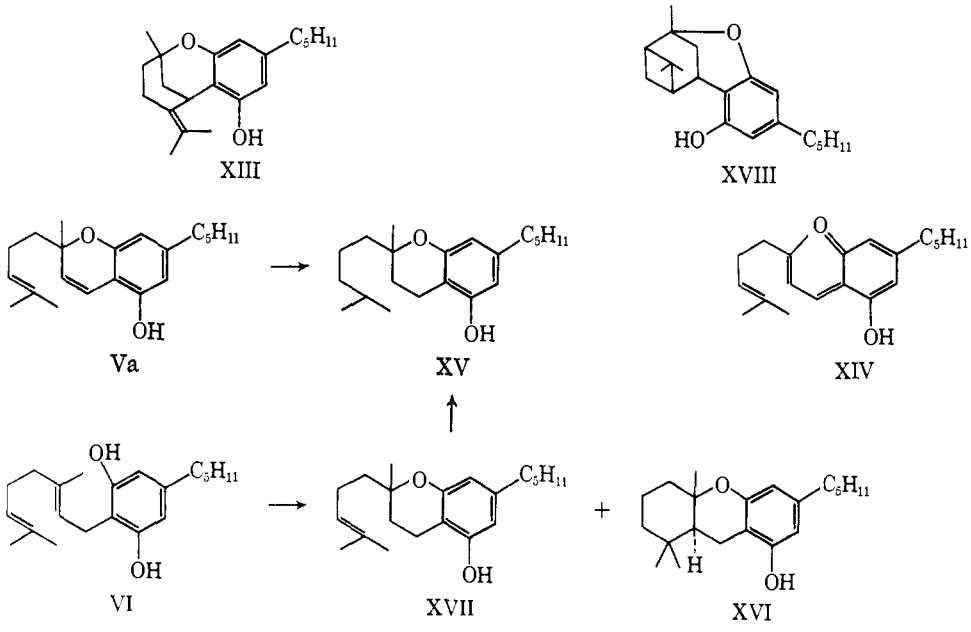
(23) R. S. de Ropp, *J. Amer. Pharm. Ass. Sci. Ed.*, **49**, 756 (1960).

(24) E. W. Gill, W. D. M. Paton, and R. G. Pertwee, *Nature*, **228**, 134 (1970).

(25) *Inter alia* H. I. Bicher, and R. Mechoulam, *Arch. Int. Pharmacodyn. Ther.*, 172, 24 (1968); H. Isbell, C. W. Gorodetzky, D. Jasinski, U. Claussen, F. von Spulak, and F. Korte, *Psychopharmacologia*, 11, 184 (1967); L. E. Hollister, R. K. Richards, and H. K. Gillespie, *Clin. Pharmacol. Ther.*, **9**, 783 (1968); C. F. Lipparini, A. S. de Carolis, and V. G. Longo, *Phys. Behav.*, **4**, 527 (1969).

(26) R. Mechoulam and B. Yagen, *Tetrahedron Lett.*, 5349 (1969).

(27) U. Claussen, F. von Spulak, and F. Korte, *Tetrahedron*, **22**, 1477 (1966).



cannabichromene it shows no rotation.[16] We assume therefore that natural cannabichromene is indeed racemic. The related cannabichromenic acid (Vb) originally reported[28a] to be optically active is in fact probably also racemic.[28b] This lack of optical activity points out that either (a) cannabichromene is an artifact, apparently formed by nonenzymic oxidation of cannabigerol, or that (b) the intermediate formed by enzymic oxidation is a symmetrical species such as XIV.

The uv spectrum of cannabichromene indicates conjugation of one of the double bonds with the ring and is compatible with the spectra of similar chromenes derived from resorcinol derivatives.[29] The nmr spectrum indicates that (a) the two aromatic protons are magnetically nonequivalent and that one of the methyl groups on the terpene moiety is α to an oxygen atom thus determining the point of attachment of the ether–oxygen atom, the other oxygen atom being in a free phenolic group; (b) two of the olefinic protons are not flanked by additional hydrogen atoms (sharp AB pattern, δ 5.44, 6.60, J_{AB} = 10 Hz); (c) the second double bond is in an isopropylidene grouping (two methyl groups, δ 1.58, 1.62, one olefinic proton, δ 5.05). These findings are compatible only with structure Va.

Cannabichromene has been correlated with cannabigerol by hydrogenation to the oily tetrahydrocannabichromene (XV), the 3,5-dinitrophenylurethane of which melts at 127–128°. The same tetrahydrocannabichromene (XV) (3,5-dinitrophenylurethane, mp 127–128°) was obtained from cannabigerol by boiling with *p*-toluenesulfonic acid in benzene to give a mixture of XVI[26] and XVII; reduction of XVII gave XV. Pure cannabichromene shows no activity in the dog ataxia or monkey behavioral tests in doses up to 10 mg/kg.[9b] The positive dog ataxia test previously observed[8c] was probably due to impurities in the natural material, which was available in minute amounts.

(28) (a) Y. Shoyama, T. Fujita, T. Yamauchi, and I. Nishioka, *Chem. Pharm. Bull.*, **16**, 1157 (1968); (b) T. Yamauchi, private communication.

(29) H. Fukami, M. Nahayama, and M. Nakajima, *Agr. Biol. Chem.*, **25**, 247 (1961); R. Ghosh, A. R. Todd, and S. Wilkinson, *J. Chem. Soc.*, 1121 (1940); G. Cardillo, L. Merlini, and R. Mondelli, *Tetrahedron*, **24**, 497 (1968).

The structure of cannabichromene has been confirmed by syntheses.[30]

Cannabicyclol (III). This minor component, mp 46°, was first isolated by Korte and Sieper.[31] It was initially[31] considered to have a THC-type structure and was named "THC III." It was later independently isolated by our group[2] and renamed cannabicyclol. The molecular weight (mass spectrum) and elementary analysis indicated the composition $C_{21}H_{30}O_2$. The uv spectrum is typical for the olivetol moiety. The nmr spectrum shows (a) two nonequivalent aromatic protons, (b) four methyl groups, none of which is olefinic, but at least one is α to an oxygen atom and one is apparently the terminal methyl group of the pentyl side chain, and (c) no olefinic protons. Apparently cannabicyclol has no double bonds. Consequently, the elemental composition requires a tetracyclic structure. Structure XVIII was suggested as a working hypothesis.[2] Parallel to our work, Claussen, *et al.*,[32] put forward XVIII as a definite structure for the same compound (now renamed cannabipinol). From a synthetic sequence Crombie and Ponsford[30b] isolated a material which was shown to be identical with cannabicyclol and to which the correct structure was assigned. A 220-MHz nmr spectrum shows that the benzylic C-3 proton is a doublet being coupled to the C-2 proton only. The only structure which fits these data is III.

The suggested constitution of cannabicyclol has received further support from an unequivocal photochemical synthesis.[33] Other syntheses have also been achieved.[30b,c,34]

Cannabicyclol shows no rotation. In view of its presumed formation from cannabichromene[33] this

(30) (a) R. Mechoulam, B. Yagnitinsky, and Y. Gaoni, *J. Amer. Chem. Soc.*, **90**, 2418 (1968); (b) L. Crombie and R. Ponsford, *Chem. Commun.*, 894 (1968); (c) V. V. Kane and R. K. Razdan, *J. Amer. Chem. Soc.*, **90**, 6551 (1968); (d) G. Cardillo, R. Cricchio, and L. Merlini, *Tetrahedron*, **24**, 4825 (1968).

(31) F. Korte and H. Sieper, *J. Chromatogr.*, **13**, 90 (1964).

(32) U. Claussen, F. von Spulak, and F. Korte, *Tetrahedron*, **24**, 1021 (1968).

(33) L. Crombie, R. Ponsford, A. Shani, B. Yagnitinsky, and R. Mechoulam, *Tetrahedron Lett.*, 5771 (1968).

(34) B. Yagen and R. Mechoulam, *ibid.*, 5353 (1969).

is not surprising. It remains to be established whether it is formed in the plant (possibly *via* a photochemical process from Va) or is formed in the resin on storage. It cannot be an artifact of the isolation and purification procedures as these are mild and do not involve steps conductive to cannabichromene cyclization.

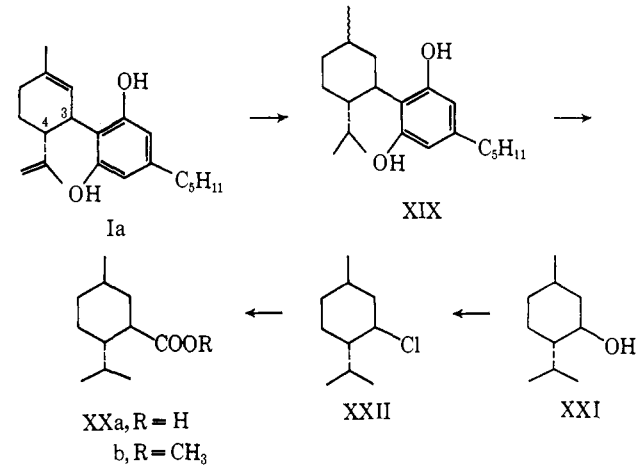
Absolute Configuration. Adams, *et al.*,[35] have reported that tetrahydrocannabidiol (XIX) obtained by reduction of cannabidiol (which has since been shown[7] to possess structure Ia) can be oxidized to the menthane carboxylic acid XXa. The anilide of XXa thus obtained did not depress the melting point of the anilide of XXa prepared from menthol (XXI) through the menthyl chloride (XXII), followed by carbonation of the Grignard derivative. However, the rotation of the anilide of XXa prepared by the degradation of the natural product was not reported.

We have repeated and extended this correlation.[8d] Catalytic hydrogenation of natural (−)-cannabidiol gave a mixture of the two C-1 epimers (XIX) which could be separated by column chromatography on Florisil. The chromatographically more polar isomer was oxidized with potassium permanganate in acetone. The acidic product obtained was esterified with diazomethane and purified by preparative vapor phase chromatography. The pure menthanecarboxylic acid methyl ester (XXb) thus obtained ([α]D −40°) was identical in all respects (ir, nmr, tlc, rotation) with XXb prepared from natural (−)-menthol (XXI) through the acid XXa[7,36] followed by methylation. Basic hydrolysis of XXb, obtained by degradation of cannabidiol, gave menthanecarboxylic acid (mp 64–65°, [α]D −44°)[36] also identical in all respects (ir, nmr, tlc, [α]D, mixture melting point) with XXa prepared from menthol (XXI).

Natural (−)-menthol has been interrelated with glyceraldehyde.[37] This correlation establishes therefore the absolute configuration of cannabidiol (Ia). As the latter has been converted into Δ^1-THC (IVa), and into $\Delta^{1(6)}$-THC (VIII), the above correlation establishes the absolute configuration of these natural products at both C_3 and C_1 as *R*. Šantavý[38] has reached similar conclusions, mainly by comparison of optical rotation data from the literature. However, some of the rotations compared were of compounds which were later shown to be mixtures.[13]

These absolute configurations were later confirmed by total syntheses which started from optically active terpenes with known chirality.[17a,c]

Analytical Aspects. In view of the practical importance of *Cannabis* analysis, numerous groups have investigated this problem. Thin layer chromatography methods are widely used for qualitative analysis. A popular procedure is the one suggested by Korte and Sieper.[31] It employs silica gel impregnated with dimethylformamide (DMF); cyclohexane is used as eluent. It has been reported, however, that the R_f values in this system are affected by the grade of dryness of the DMF.[39]



Numerous other solvents have also been employed.[39,40] In our investigations we have used a rather simple system: chromatoplates of silica gel; elution with petroleum ether (bp 40–60°)–ether in a ratio of 8:2. The plates were sprayed with a potassium permanganate solution. The R_f values of the major natural neutral cannabinoids are tabulated in Table I. Vapor phase chromatography has been extensively employed. The columns in use today are SE 30,[10,41] XE 60,[42] Carbowax 20 M,[43] OV 17.[11,44] We have routinely used 2% OV-17 on Chromosorb Q at 235°. The retention times of the major natural neutral cannabinoids are tabulated in Table I. It should be pointed out that all cannabinoid acids undergo decarboxylation at the high temperatures employed for vpc (200–250°). For a routine analysis this may be an advantage, for this reaction parallels the smoking process. A vpc analysis will thus give directly all the THC available on smoking in a certain sample. When an exact determination of the content is required, decarboxylation can be prevented by esterification.

Experimental Section

General. The ir spectra were recorded on a Perkin-Elmer Model 137 instrument, the nmr spectra were measured on a Varian A-60 spectrometer, and the uv spectra were measured on a Cary 14 spectrometer. The ir curves of the natural neutral cannabinoids described in this paper have been reproduced.[2] Detailed uv and nmr spectra of these compounds have been described.[2] Most of the mass spectra have been reported.[45] The remaining mass spectra were measured on an Atlas CH4 instrument: tlc, chromatoplates of silica gel G (Merck), elution with petroleum ether (bp 40–60°) and ether in a ratio of 4:1; developer, 0.5% potassium permanganate in a saturated solution of cupric acetate. Vapor phase chromatography was conducted on a Packard Model 803 with a flame ionization detector, glass columns (6 ft × 1/8 in.) with 2% OV-17 on Gas Chrom Q, N_2 flow rate 30 cm³/min, column temperature 235°. The microanalyses were performed by the microanalytical department of the Weizmann Institute.

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Extraction of Hashish. Hashish "soles" of undetermined age (not less than 1 year old) were obtained from police sources. They originated from various "producers," presumably in Lebanon, as indicated by the stamped markings on their cloth covers. Each weighed *ca.* 200 g. Two "soles" (402 g) were broken into small pieces and stirred mechanically with 4 l. of petroleum ether (bp 60–80°) at room temperature in a glass vessel. The mixture was filtered with suction, the extraction was repeated twice, and the extracts were combined. A total of 135 g of extract was obtained, as determined by evaporating an aliquot to dryness under high vacuum and weighing the dry oil. The dark brown petroleum ether solution was concentrated to *ca.* 1 l. and was extracted three times with 250 ml of an aqueous solution of 5% sodium hydroxide and 2% sodium sulfite in the presence of ice. The aqueous phase was worked up as described before[12b] to yield 28 g of acids (6.96% of hashish). The petroleum ether layer was washed with a saturated solution of sodium chloride (200 ml), dried over sodium sulfate, and evaporated to give 65 g (16.2% hashish) of a dark viscous oil. In addition to the two liquid phases a dark tar (21 g, 5.2% hashish) was deposited on the walls of the vessels during the separation of the neutral and acidic components. Our experience over the last few years has been that the petroleum ether soluble acids represent 5–10% of hashish, the neutral, petroleum ether soluble fraction 13–22%, and the above described tar 3–6%.

Since no two extracts were found to have exactly the same composition, chromatographic separation was best carried out in stages. A first rough fractionation was effected by chromatography on alumina (Merck, acid washed) or on Florisil, the components being eluted with increasing proportions of ether in pentane.

In a typical experiment, 25 g of the crude, neutral oily material was charged onto a column of 500 g of Merck acid-washed alumina. Fractions of 150 ml were collected. Each fraction was examined by tlc and vpc; fractions with similar composition were combined. The following crude separation was achieved: (A) waxy, noncannabinoid materials, 0.4 g, fractions 1–15 (eluted with pentane); (B) cannabidiol (mainly), 3.9 g, fractions 16–29 (eluted with pentane–ether, 98:2); (C) cannabidiol (mainly), cannabicyclol, Δ^1-THC, 3.5 g, fractions 30–40 (eluted with pentane–ether, 98:2); (D) Δ^1-THC, cannabinol, 4.1 g, fractions 47–62 (eluted with pentane–ether 95:5); (E) cannabinol, cannabichromene, cannabigerol, 4.4 g, fractions 63–78 (eluted with pentane–ether 92:8 to 85:15); (F) polar noncannabinoid substances, 1.6 g, fractions above 79 (eluted with pentane ether 3:1 and 1:1).

The fractions containing cannabinoids B–E were rechromatographed several times to achieve separation of the individual components. Typical chromatographies are described below; the following total yields (in grams from the above experiment, as per cent of hashish) of *isolated* compounds were recorded: cannabicyclol (III) (0.127 g, 0.082%), cannabidiol (Ia) (3.94 g, 2.54%), Δ^1-THC (IVa) (3.25 g, 2.10%), cannabinol (II) (1.27 g, 0.82%), cannabichromene (Va) (0.156 g, 0.102%), and cannabigerol (VI) (0.326 g, 0.21%). The amounts of neutral cannabinoids in the same batch, as determined by vpc, were higher (as per cent of hashish): cannabicyclol (0.11%), cannabidiol (3.74%), Δ^1-THC (3.30%), cannabinol (1.3%), cannabichromene (0.19%), and cannabigerol (0.3%).

Cannabicyclol (III). This compound accompanies most of the cannabidiol fractions in the first rough separation. It can be separated from the latter by chromatography on alumina coated with 12% by weight of silver nitrate. Alumina thus treated reverses the order of elution mentioned above. Rechromatography of a portion of the above fraction B (3.0 g) on 300 g of alumina–silver nitrate yielded, in order of elution: cannabicyclol (III), 75 mg (4% ether in pentane); cannabinol (II), 167 mg (10% ether); Δ^1-THC (IVa), 460 mg (10% ether); cannabidiol (Ia), 1.20 g (20% ether). Cannabicyclol was obtained as a solid: mp 146–147° (pentane);[46] mol wt (mass spectrum) 314; λ_{max} (C_2H_5OH) 275 (ϵ 1240) and 282 mμ (ϵ 1270); nmr, δ ($CDCl_3$) 0.80, 0.90, 1.38 (four CH_3), 3.12 (br d, C-3 H), 6.18, 6.33 (aromatic H).

Anal. Calcd for $C_{21}H_{30}O_2$: C, 80.21; H, 9.62. Found: C, 80.34; H, 9.68.

Cannabidiol (Ia).[7,47] Cannabidiol, the main neutral cannabinoid, was obtained as a solid, mp 66–67°. It gave the known bis-3,5-dinitrobenzoate,[47] mp 106–107°, and a bis-*p*-toluenesulfonate,[18a] mp 81–83°.

Anal. Calcd for $C_{35}H_{42}O_6S_2$: C, 67.51; H, 6.80. Found: C, 67.52; H, 6.75.

Δ^1-Tetrahydrocannabinol (IVa). Δ^1-THC was obtained in over 95% purity by repeated chromatography of enriched fractions. Thus, in one case, a crude fraction of 2.5 g containing mainly cannabidiol and Δ^1-THC (by tlc and vpc) was rechromatographed on alumina (250 g) yielding 1.13 g of cannabidiol and 1.20 g of *ca.* 80% pure Δ^1-THC. The latter was combined with a crude fraction of Δ^1-THC of equal purity and the total (4.7 g) was rechromatographed on 350 g of alumina. For elution ether in pentane was used and 100-ml fractions were collected. Alternating fractions were evaporated at reduced pressure and the oily product was checked by vpc, tlc, and eventually ir. A total of 0.3 l. of pentane, 3 l. of 5% ether, 1.5 l. of 10% ether, and 1 l. of 20% ether in pentane were used to elute 3.9 g of material, of which 3.0 g was pure Δ^1-THC. It was obtained as a colorless oil, coloring rapidly in air, in the absence of solvent, with a violet brownish tint, but retaining its spectral and analytical properties, as well as its biological activity.

A 3,5-dinitrobenzoate was prepared from 156 mg of Δ^1-THC and 200 mg of 3,5-dinitrobenzoyl chloride in pyridine, followed by chromatography on silica gel (elution with 2% ether in pentane). This yielded a homogenous product by tlc, which could not be induced to crystallize; $[\alpha]D$ −71°.

Anal. Calcd for $C_{28}H_{32}N_2O_7$: C, 66.13; H, 6.34. Found: C, 66.41; H, 6.22.

A 3,5-dinitrophenylurethane was prepared by boiling 1.5 g of Δ^1-THC with 1.5 g of 3,5-dinitrobenzoyl azide in 50 ml of toluene for 4 hr. The mixture was cooled overnight and insoluble material was filtered. Toluene was evaporated at reduced pressure and the residue was taken up in benzene; the 3,5-dinitrophenylurethane of cannabinol may precipitate at this stage, if cannabinol accompanied the Δ^1-THC used; it is filtered off. The benzene solution was chromatographed on silica gel (250 g) and elution was carried out with 5, 10, and 20% ether in pentane. Ether (10%) in pentane eluted two solid materials. The first solid (0.6 g) was recrystallized from pentane and identified as the **3,5-dinitrophenylurethane of Δ^1-THC** (VIIa), mp 113–115°.

Anal. Calcd for $C_{28}H_{33}N_3O_7$: C, 64.23; H, 6.35. Found: C, 64.17; H, 6.54.

The second solid (0.4 g) was recrystallized from hexane and identified as the **amide VIIb:** mp 145–146°; δ ($CDCl_3$) 0.9, 1.04, 1.38 ($-CH_3$ groups), 1.60 (olefinic CH_3 group), 3.12 (br d, C-3 H), 6.05 (s, aromatic H), under the aromatic H, at 6.10 (br, C-2 proton), 8.20 (amide H), 8.90 (3 H, aromatic), 10.4 (hydrogen bonded OH, exchangeable with D_2O).

Anal. Calcd for $C_{28}H_{33}N_3O_7$: C, 64.23; H, 6.35. Found: C, 64.45; H, 6.61.

Hydrolysis of the 3,5-Dinitrophenylurethane of Δ^1-THC (VIIa) to Δ^1-THC. The urethane (300 mg) was dissolved in ethanol, a 10% excess of the calculated amount of 10% hydroalcoholic potassium hydroxide solution was added, and the solution was warmed in a water bath at 60–70° for 10 min. Water was added to the cooled solution which was then extracted with pentane. The pentane extract was washed with water and dried over sodium sulfate. Evaporation of the pentane and redissolution in a small amount of the same solvent left some insoluble dinitroaniline, which was filtered. Chromatography of the pentane solution on alumina yielded pure Δ^1-THC. It was distilled in a bulb-to-bulb distillation apparatus, bp *ca.* 220° (bath temperature) (0.1 mm). This compound did not differ by any of the standard criteria of purity from the product as obtained after repeated chromatography (ir, uv, nmr, tlc, $[\alpha]D$), $[\alpha]D$ −150° ($CHCl_3$); mol wt (mass spectrum)[45] 314; λ_{max} (C_2H_5OH) 277 (ϵ 1640), 282 mμ (ϵ 1550); δ (CCl_4) 0.88, 1.08, 1.38 (CH_3 groups), 1.68 (olefinic CH_3), 3.14 (br d, C-3 H), 6.00 (d, J = 2 Hz, aromatic H), 6.18 (d, J = 2 Hz, aromatic H), 6.35 (br s, C-2 H).

Anal. Calcd for $C_{21}H_{30}O_2$: C, 80.21; H, 9.62. Found: C, 80.20; H, 9.55.

Dehydrogenation of Δ^1-THC (IVa) to Cannabinol (II). A mixture of 170 mg of Δ^1-THC (IVa) and 40 mg of sulfur was heated at *ca.* 250° for 45 min. It was dissolved in benzene and filtered through a small column of silicic acid. The oil was distilled in a bulb-to-bulb apparatus, bp *ca.* 200 (bath temperature) (0.1 mm). The compound obtained was identical with cannabinol isolated from hashish (ir, nmr, tlc, vpc). The acetate of II, mp 76–77°, from dehydrogenation, did not depress the mp of II, 76–77°, from hashish.

(46) When first isolated by us cannabicyclol (III) was found[2] to melt at 152–153°. On subsequent isolations or on synthesis the mp was 145–146°.

(47) R. Adams, M. Hunt, and J. H. Clark, *J. Amer. Chem. Soc.*, **62**, 196 (1940).

Cannabinol (II) from Hashish. This constituent[2,48] was obtained as described above by rechromatography of crude fractions containing Δ^1-THC and cannabinol. While it is possible to obtain crystalline II, mp 75–76°, it is more easily identified as the acetate, mp 76–77°.

A **3,5-dinitrophenylurethane of II** was prepared as described above. It melts at 233–234° (hexane–ether).

Anal. Calcd for $C_{28}H_{29}N_3O_7$: C, 64.73; H, 5.63. Found: C, 64.47; H, 5.74.

Cannabichromene (Va) and Cannabigerol VI. Repeated chromatography of crude fraction E was necessary in order to get the oily minor constituent Va in pure form. It is eluted from Florisil with 3–5% ether in pentane, following cannabinol and preceding cannabigerol (VI). Purity was monitored by tlc, vpc, and nmr. Pure fractions were combined. Distillation in a bulb-to-bulb apparatus yielded the pure compound: bp 220° (bath temperature) (0.1 mm); mol wt (mass spectrum)[45] 314; λ_{max} (C_2H_5OH) 228 (ϵ 25,100) and 280 mμ (ϵ 8900); δ (CCl_4) 0.87, 1.32 ($2CH_3$), 1.58, 1.62 (2 olefinic CH_3), 5.05 (br tr, olefinic H), 5.44, 5.60 (AB quartet, J_{AB} = 10 Hz, 2 olefinic H), 5.97, 6.15 (2 aromatic H).

Anal. Calcd for $C_{21}H_{30}O_2$: C, 80.21; H, 9.62. Found: C, 80.00; H, 9.47.

The **3,5-dinitrophenylurethane of cannabichromene** was prepared as for Δ^1-THC above, mp 106–107° (hexane).

Anal. Calcd for $C_{28}H_{33}N_3O_7$: C, 64.23; H, 6.35; N, 8.03. Found: C, 64.06; H, 6.31; N, 8.38.

Pure **cannabigerol** (VI) was obtained in the fraction following cannabichromene and is recognized by being a solid. It was purified by recrystallization from pentane: mp 51–53°; mol wt (mass spectrum)[45] 316; λ_{max} 272 (ϵ 1100) and 280 mμ (ϵ 1050); δ (CCl_4) 0.95 (CH_3), 1.60, 1.69, and 1.82 (3 olefinic CH_3), 3.35 (2 H, d, J = 7.5 Hz, C-8 H), 4.90–5.35 (mult, 2 olefinic H), 6.18 (2 aromatic H).

Anal. Calcd for $C_{21}H_{32}O_2$: C, 79.70; H, 10.19. Found: C, 79.84; H, 10.41.

Isomerization of Cannabidiol (Ia) to Δ^1-THC (IVa) with Boron Trifluoride. To a solution of Ia (4.8 g) in methylene chloride (200 ml), 1 ml of boron trifluoride etherate was added with stirring and the solution was set aside at room temperature for 30 min. Ether (200 ml) and water (200 ml) were added; the organic layer was separated and washed with sodium bicarbonate solution and with saturated sodium chloride solution, and dried over sodium sulfate. An nmr examination of the crude oily mixture obtained by evaporation of the solvent showed a terminal methylene to vinylic hydrogen ratio of 1:2. Separation of the mixture was effected by chromatography on Florisil (500 g) and elution was carried out with pentane and with increasing portions of ether in pentane, a total of 15 l. of solvent being used. Alternating fractions of 250 and 100 ml were collected; the 100-ml fractions were evaporated under reduced pressure and examined by tlc, ir, and nmr. The first compound to be eluted (0.66 g; 1% ether in pentane), showing one spot on tlc, was identified as Δ^8-*i*-THC (IX).[16] The following fraction, eluted with the same mixture of solvents, showed two spots on tlc corresponding to IX and IVa (0.6 g) and then IVa (1.94 g) showing only 3–4% of IX by vpc. Pure IVa (0.94 g) was then eluted (2–4% ether in pentane) identical in all respects with the natural Δ^1-THC (IVa).[8b] In a few instances, probably due to prolonged acid treatment, the formation of $\Delta^{1(6)}$-THC (VIII) was observed. This compound was then eluted with IX from which it could be separated by chromatography on alumina coated with silver nitrate.

Tetrahydrocannabichromene (XV) was prepared by catalytic hydrogenation of 250 mg of cannabichromene with Adams catalyst (25 mg) at atmospheric pressure in 5 ml of ethyl acetate. After *ca.* 5 ml of hydrogen was absorbed, the catalyst was filtered off, ether was added, and the solution was washed with water, sodium bicarbonate solution, and water, and dried. Removal of the solvent yielded an oil, which showed one peak on vpc. After distillation in a bulb-to-bulb apparatus the oil showed λ_{max} (C_2H_5OH) 281 (ϵ 1250), 275 mμ (ϵ 1230); $[\alpha]D$ 0°; δ (CCl_4) 0.82, 0.92, 1.20 (CH_3 groups), 5.98, 6.10 (2 H, aromatic), no olefinic protons or olefinic methyl groups.

Anal. Calcd for $C_{21}H_{34}O_2$: C, 79.19; H, 10.76. Found: C, 79.43; H, 11.02.

The **3,5-dinitrophenylurethane** of XV melts at 127–128° (hexane).

Anal. Calcd for $C_{28}H_{37}N_3O_7$: C, 63.74; H, 7.07; N, 7.97. Found: C, 63.77; H, 7.24; N, 7.98.

Dihydrocannabichromene (XVII) and the Tricyclic XVI. A solution of cannabigerol (0.22 g) and *p*-toluenesulfonic acid (20 mg) in benzene (50 ml) was boiled for 30 min. The solution was washed with 5% sodium bicarbonate solution, dried, and evaporated. The mixture was chromatographed on 15 g of acid-washed alumina containing 12% silver nitrate. Elution with pentane–ether (99:1) gave an oil (XVI) (116 mg) which on vpc (2% OV 17 on Gas Chrom Q at 230°) showed one peak. A small amount of the cis isomer[26] was present (by vpc) in some of the early fractions but was not isolated. The cis isomer is the major product of the acid cyclization of 6-*cis*-cannabigerol[26,34] and is clearly distinguishable from the trans isomer. The trans isomer XVI has λ_{max} (C_2H_5OH) 274 (ϵ 1060), 281 mμ (ϵ 1050); δ (CCl_4) 0.95, 1.05, 1.25 (4 CH_3 groups), 6.10, 6.20 (2 aromatic protons); mol wt (mass spectrum) 316.

Anal. Calcd for $C_{21}H_{32}O_2$: C, 79.70; H, 10.19. Found: C, 79.82; H, 10.15.

The **3,5-dinitrophenylurethane** of XVI melts at 130–131° and then at 146–147° (benzene–pentane).

Anal. Calcd for $C_{28}H_{35}O_7N_3$: C, 63.99; H, 6.71; N, 7.99. Found: C, 64.22; H, 6.42; N, 7.90.

Elution with pentane–ether (99:2) gave 0.65 g of **dihydrocannabichromene** (XVII), λ_{max} (C_2H_5OH) 276 (ϵ 1010) and 282 mμ (ϵ 1030); δ (CCl_4) 0.88, 1.25 (2 CH_3, groups), 1.60, 1.65 (2 olefinic CH_3 groups), 5.0 (olefinic H), 6.00, 6.12 (2 aromatic H); mol wt (mass spectrum) 316.

Anal. Calcd for $C_{21}H_{32}O_2$: C, 79.70; H, 10.19. Found: 79.62; H, 10.02.

The **3,5-dinitrophenylurethane** of XVII melts at 97° (cyclohexane–pentane).

Anal. Calcd for $C_{28}H_{35}O_7N_3$: C, 63.99; H, 6.71; N, 7.99. Found: C, 64.12; H, 6.71; N, 8.15.

Tetrahydrocannabichromene (XV) was prepared from XVII by catalytic hydrogenation as described for the reduction of cannabichromene to XV. The nmr, ir, and uv spectra as well as the vpc and tlc behavior of XV from both reactions are identical. The 3,5-dinitrophenylurethane, mp 127–128°, of XV prepared from XVII does not depress the melting point of XV prepared from cannabichromene.

Conversion of Cannabidiol into Menthanecarboxylic Acid (XXa). Tetrahydrocannabidiol (XIX) was obtained by hydrogenation of cannabidiol (Ia) (3 g) in ethanol (20 ml) with platinum black as catalyst. After 1 hr the catalyst was filtered off, the solvent was evaporated, and, as the oil obtained still showed the presence of an olefinic proton in the nmr, the reduction was repeated. The product was chromatographed on 150 g of Florisil. Elution with 2% ether in pentane yielded two fractions. The less polar one (600 mg) was shown by vpc to be a mixture. The nmr spectrum of this mixture showed no olefinic protons or methyls, indicating that it probably consists of the two C-1 epimers of tetrahydrocannabidiol. The more polar fraction (1.2 g) was identified as XIX[35] on the basis of its nmr spectrum, δ (CCl_4) 0.75, 0.88 (CH_3 groups), 6.0 (2 aromatic H), no olefinic protons of methyl groups. It was oxidized without further purification. Potassium permanganate (2.5 g) was added to a solution of 600 mg of XIX in 180 ml of acetone. The mixture was stirred for 3 hr, after which 20 ml of a 5% HCl solution and dry sodium bisulfite were added until a clear solution was obtained. Ether (200 ml) was added. The organic layer was extracted with 5% sodium bicarbonate solution, which was washed with ether and acidified with 10% sulfuric acid. The cloudy solution was extracted with ether, which was dried and evaporated. The residue had a strong fatty acid smell. It was treated with excess diazomethane. The oily ester was purified by preparative vpc (at 150° on a 0.2% Apiezon L on glass beads). The pure **menthanecarboxylic acid methyl ester** (XXb) which was collected (170 mg) showed ν_{max} ($CHCl_3$) 1725 cm^{-1}; δ (CCl_4) 0.70, 0.82, 0.92 (3 CH_3 groups), 3.58 (–$COOCH_3$ groups); $[\alpha]D$ (C_2H_5OH) –40°. The methyl ester XXb (20 mg) was hydrolyzed by boiling for 1 hr with a 5% solution of sodium hydroxide in ethanol–water (1:1). Water and ether were added and the aqueous layer was acidified (10% sulfuric acid), extracted with ether, dried, and evaporated. The **menthanecarboxylic acid** (XXa) obtained was recrystallized to give pure XXa, mp 65–66°, $[\alpha]D$ (C_2H_5OH) –50°; ν_{CCl_4} 1700 cm^{-1}. Both the ester (XXb) and the acid (XXa) were identical in all respects (vpc, tlc, ir, nmr, $[\alpha]D$, mmp for XXa) with XXb and XXa prepared from (–)-menthol (XXI) *via* menthyl chloride (XXII), through the Grignard derivative followed by carbonation to XXa

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and methylation to XXb.[7,36,49] The relative and absolute configurations of these compounds have been established.[7,36]

Acknowledgments. We thank Professor H. Edery and Dr. Y. Grunfeld of the Biological Institute, Ness-Ziona, Israel for the psychopharmacological tests, Dr. N. Danieli for the mass spectra, Miss I. Ramati and Mrs. N. Shoef for their excellent technical help, and the Israeli Police for the supply of confiscated hashish. The last stages of the above research were supported by the National Institute of Mental Health (Grant No. MH-13180) whom we thank.

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Studies of the Chymotrypsinogen A Family of Proteins. VIII. Thermodynamic Analysis of Transition I of the Methionine Sulfoxide Derivatives of α-Chymotrypsin[1]

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Abstract: Spectral changes at 293 nm have been used to monitor the first thermal-unfolding transition (transition I) of the monomethionine sulfoxide and dimethionine sulfoxide derivatives of α-chymotrypsin. From these data ΔF°, ΔH°, and ΔS° have been calculated as a function of pH and temperature. Monomethionine sulfoxide chymotrypsin and its parent, chymotrypsin, show identical transition I characteristics. On the other hand, dimethionine sulfoxide chymotrypsin is thermodynamically less stable than its parent although transition I still exhibits all-or-none cooperativity. The thermodynamic results are in complete accord with the predictions of Brandts' "force" analysis of protein unfolding and provide strong support for this type of analysis. For example, dimethionine sulfoxide chymotrypsin exhibits a temperature of maximum stability which is a characteristic consequence of a change in the number of interactions between water and the nonpolar moieties of the protein which occurs on unfolding. Parameters of Brandts' analysis of transition I evaluated with the aid of model compound data allow comparisons among α-chymotrypsin, chymotrypsinogen, and dimethionine sulfoxide α-chymotrypsin to be made. The cooperative unfolding units of dimethionine sulfoxide α-chymotrypsin and chymotrypsinogen are approximately one-half that of α-chymotrypsin. The results are consistent with results obtained by other investigators using nuclear magnetic resonance line widths as a measure of segmental flexibility and calorimetric measurements of enthalpy changes and heat capacity. It appears that the thermally unfolded states of all the chymotrypsin proteins thus far studied are very similar, although a significant amount of folded structure is retained in this state. Since the cooperative unfolding unit of dimethionine sulfoxide chymotrypsin is only about half that of its parent, this protein must be partially unfolded in its best folded state. The change in enzymic efficiency of dimethionine sulfoxide chymotrypsin may be related to this partial unfolding which apparently must be restored before chemical catalysis can take place.

Although chemical and quantitative understanding of the unfolding processes of small globular proteins is very incomplete, some important phenomenological details having general significance have been established. Specifically we may list: (1) as first noted by Brandts,[2,3] these transitions are marked by large heat-capacity changes so that the van't Hoff isochores have marked curvature and may in some cases show a maximum in free energy of unfolding. In such cases unfolding can be produced by lowering as well as raising the temperature from the "temperature of maximum stability." (2) Some proteins demonstrate simple "two-state" behavior[4] which means that only two macroscopic states need be considered, the folded state, hereafter called state A, and the unfolded state, state B.[5,6] (3) Unfolding to the completely unfolded state need not occur in a single step. Ribonuclease A[7,8] and α-chymotrypsin[9-11] on increasing temperature experience two-state transitions which produce only a partial unfolding. Complete unfolding of chymotrypsin has not been effected by raising the temperature although it occurs in 8 *M* urea.[12] (4) The dominant characteristic of two-state transitions is the high degree of cooperativity but proteins of the same or nearly the same amino acid sequence, such as chymotrypsinogen A (CGN) and α-chymotrypsin (CT),[10] can have different numbers of

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(1) This is publication No. LBC 33. Request reprint by number. The work was supported by the U. S. Public Health Service through National Institutes of Health Grant No. 2 R01 AM 05853 and by fellowship support from the 3M Corporation to R. B. This work is reported in full in the Ph.D. Dissertation of R. Biltonen, University of Minnesota, 1965. A preliminary report of this work has appeared (R. Biltonen and R. Lumry, *J. Amer. Chem. Soc.*, 87, 4208 (1965)).

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