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Dioxygenated metabolites of cannabidiol formed by rat liver

BILL MARTIN*, STIG AGURELL*†, MARIANNE NORDQVIST*§, AND JAN-ERIK LINDGREN†‡

* *Department of Pharmacognosy, Faculty of Pharmacy, BMC, Box 579, S–751 23 Uppsala, Sweden,* † *Astra Läkemedel AB, S–151 85 Södertälje, Sweden, and* ‡ *Department of Toxicology, Swedish Medical Research Council Karolinska Institute, S–104 01 Stockholm, Sweden*

The metabolism of cannabidiol (CBD) was studied *in vitro* using a 10 000 *g* supernatant from rat liver. After removal of unchanged CBD and its monohydroxylated metabolites, a polar fraction remained from which ten dioxygenated metabolites were isolated. Mass spectrometry and nuclear magnetic resonance spectroscopy were used to identify the following metabolites: 6,7-dihydroxy-CBD, 1″,7-dihydroxy-CBD, 3″,7-dihydroxy-CBD, 4″,7-dihydroxy-CBD, 5″,7-dihydroxy-CBD, 2″,6-dihydroxy-CBD, 3″,6β-dihydroxy-CBD, 4″,6β-dihydroxy-CBD (tentative), 3″-hydroxy-6-oxo-CBD, and 4″-hydroxy-6-oxo-CBD. The abundance of isolated dihydroxy metabolites reflected the quantity of monohydroxy metabolites that was previously found. In both series, 7-hydroxylation occurred to the greatest extent. Side chain hydroxylation occurred predominantly at C-4″ and to a lesser degree at C-3″. Trace amounts of metabolites were hydroxylated at C-1″, -2″, or 5″.

CBD has no behavioural effects in man (Perez-Reyes, Timmons & others, 1973), but it does appear to alter the effects of Δ^1-tetrahydrocannabinol (Δ^1-THC) in animals (Karniol & Carlini, 1972) and in man (Hollister, 1973; Karniol, Shirakawa & others, 1974). It has been suggested that CBD may be interfering with the metabolism of Δ^1-THC. Indeed, Jones & Pertwee (1972) reported that CBD interfered with the *in vivo* metabolism of both Δ^1-THC and 7-hydroxy-Δ^1-THC. CBD's actions could be explained by the fact that it has a greater affinity for the microsomal enzymes than does Δ^1-THC (Bailey & Toft, 1973). Therefore, it was not surprising that both cannabinoids were monohydroxylated in a similar way (Martin, Nordqvist & others, 1976), the primary exception being that CBD was more diversely metabolized. Likewise, further metabolism of 7-hydroxy-Δ^1-THC could be impeded by the conversion of 7-hydroxy-CBD to dihydroxy metabolites.

In the THC series both 7-hydroxylation (Truitt, 1971; Christensen, Freudenthal & others, 1971) and side chain hydroxylation (Agurell, Binder & others, 1976) enhance pharmacological activity. By analogy with the THC series the anticonvulsant activity of CBD (Carlini, Leite & others, 1973; Izquierdo & Tannhauser, 1973) could be enhanced by hydroxylation in these positions. The purpose of the present investigation was to determine which dihydroxylated metabolites of CBD, if any, were formed *in vitro* by rat liver enzymes.

§ Correspondence.

MATERIALS AND METHODS

In vitro metabolism of radiolabelled CBD

(−)-1″-[^{3}H]-CBD was synthesized as described by Agurell, Gustafsson & others (1973). The radiochemical purity was determined to be 98% by g.l.c. and t.l.c. The final specific activity was adjusted to 0·15 m Ci mmol^{-1} by the addition of non-labelled (−)-CBD.

Male Sprague-Dawley rats (n = 20; 200 g) were treated with sodium pentobarbitone (40 mg kg^{-1}, i.p. twice daily for four days) and then decapitated. Their livers (22·9 g) were removed, homogenized in isotonic KCl (2 vol), and subsequently centrifuged at 10 000 *g*. The microsomal containing supernatant was enriched with appropriate cofactors (Jones, Widman & others, 1974) before the addition of 125 mg of radiolabelled CBD. The incubation was at 37° for 2 h and 45 min.

Isolation of metabolites

The incubate was extracted with light petroleum (40–60°, 3 × 650 ml) and with diethyl ether (ether) (3 × 500 ml). Metabolites in the combined ether extracts were separated on a Florisil column (115 g) by increasing the polarity of the eluent as shown in Fig. 1. The 5% methanol–ether fraction was further chromatographed on a Sephadex LH-20 column (Widman, Nordqvist, & others, 1974). The final separation of these metabolites was carried out on t.l.c. plates that had been prewashed with benzene–methanol (1:1).

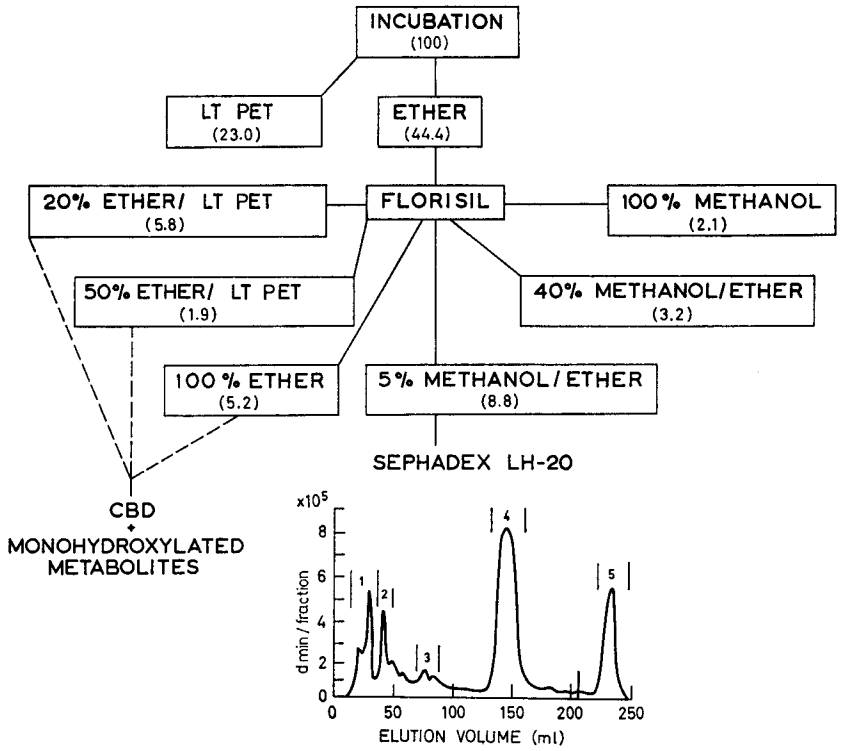

FIG. 1. Scheme for isolation of CBD metabolites. Numbers in parentheses are the percent of total radioactivity. Metabolites were eluted from the Florisil column with 500 ml of each solvent in the order of increasing polarity. The Sephadex LH-20 column was eluted with 210 ml of light petroleum–chloroform–ethanol (10 : 10 : 1) which was followed by a methanol wash. Two ml fractions were collected, counted for radioactivity (d min^{-1}/2 ml fraction on the ordinate) and combined to give the five Sephadex LH-20 fractions as shown.

Thin-layer chromatography and identification methods

Precoated Silica Gel F plates (Merck 0·25 mm thickness, 5 × 10 cm) were used for t.l.c. The plates were developed in methanol–ether (1% v/v unless stated otherwise), and the metabolites were visualized with 0·1% Fast Blue B salt in 2 N sodium hydroxide. Radioactivity was assayed in a Packard Tricarb model 3375 spectrometer with external standardization. Using a Varian Aerograph Model 2100, g.l.c. was carried out on a 2% SE-30 column (Gas-Chrom Q) at 250°. Before g.l.c. analysis, all samples were silylated with *N,O*-bis-(trimethylsilyl)-acetamide in dry acetonitrile. An LKB 9000 gas chromatograph - mass spectrometer (LKB-Produkter, Bromma, Sweden) was used to record all mass spectra (m.s.). The column was 3% SE-30 on Gas-Chrom (190°). M.s. of the silylated derivatives were recorded at 20 eV while the m.s. of non-derivatized samples were determined at 70 eV. ^{1}H-nuclear magnetic resonance (^{1}H-nmr) spectra were recorded by a Varian 100 MHz instrument ($CDCl_3$, Fourier Transform).

RESULTS

Following the incubation of CBD with the rat liver supernatant, unchanged CBD and its metabolites were isolated as outlined in Fig. 1. Light petroleum extraction removed predominantly CBD, while the ether extraction that followed removed neutral metabolites as well as any residual CBD. The ether extract was chromatographed on a Florisil column, and the first eluate contained starting material. The two subsequent eluates were composed of monohydroxylated metabolites as described earlier (Martin & others, 1976). The 5% methanol–ether eluate contained more polar metabolites, most of which did not move from the origin on t.l.c. (60% ether light petroleum). These metabolites were isolated after separation on a Sephadex LH-20 column. Five fractions were obtained as indicated in Fig. 1.

Fraction 1 represented 3% of the radioactivity in the ether extract but was not further investigated, since it was highly contaminated with lipid material.

Fraction 2 was chromatographed on preparative

t.l.c. plates (100% ether) which allowed the isolation of metabolite I [118 μg, R_F = 0·57 in chloroform–acetone (13:7), retention time (Rt) = 2·9 min]. The m.s. of the silylated metabolite indicated that both hydroxylation and hydration had occurred. A structural assignment could not be made, although the distinctive fragment *m/e* 337 showed that oxygenation had occurred only in the monoterpene moiety.

Fraction 3 was chromatographed further on preparative t.l.c. (100% ether) which permitted the isolation of a band that contained a single metabolite (II). Trace quantities of metabolite II (Table 1) also appeared in fraction 5. M.s. (relative intensities) showed *m/e* 560 (23, M^+), 545 (12), 532 (9), 492 (80), 425 (100), 391 (21), and 117 (17). The 560 (M^+) was consistent with trisilylation of a hydroxy-oxo-metabolite. After comparison of the m.s. fragmentation pattern to those of monooxygenated CBD metabolites, metabolite II was assigned a structure of 4″-hydroxy-6-oxo-CBD. The basis for the structural assignments of this and other compounds is presented in the discussion. Identification of other metabolites in this fraction was not possible due to their occurrence in trace amounts.

Fraction 4 (3·5 mg) was identified as 7-hydroxy-CBD (Martin & others, 1976).

Fraction 5 (2·4 mg) was a complex mixture of metabolites that were separated on preparative t.l.c. Metabolites were present in seven distinct bands (R_F = 2·8, 4·9, 6·3, 6·7, 8·6, 9·1, 9·6) that were eluted from the plates.

Band 1 (R_F = 2·8) contained a polar dihydroxy metabolite but structural elucidation by m.s. was not possible.

Band 2 contained three metabolites (III–V) which were partially separated by t.l.c. (1% methanol–ether, 3 times). This chromatographic procedure was repeated a further three times before the metabolites were isolated in pure form. Metabolite III had R_F = 0·28 and Rt = 3·8 min. A fully silylated dihydroxy metabolite-(III) was indicated by *m/e* 634 (5, M^+), 566 (100), 531 (6), 478 (13), 337 (26), 147 (22), and 103 (10), probably 6,7-dihydroxy-CBD. Metabolite IV was responsible for the major portion of radioactivity (Table 1). Prominent m.s. fragments were *m/e* 634 (15, M^+), 566 (70), 531 (100), 441 (10), 425 (39), 117 (21), and 103 (16). The ^{1}H-nmr spectrum had signals at δ ($CDCl_3$), 6·30 (s, 2H, C-3′, C-5′), 5·85 (s, br, 1H, C-2), 4·65 and 4·57 (s, 1H, C-9), 4·12 (s, 2H, C-7), 2·49 (t, 2H, C-1″), 1·67 (s, 3H, C-10) and 1·19 (d, 3H, C-5″). On the basis of these data, metabolite IV was determined to be 4″,7-dihydroxy-CBD. The third metabolite (V) isolated from band 2 was found to be 5″,7-dihydroxy-CBD. It had R_F = 0·18, Rt = 7·2 min and m.s. fragments *m/e* 634 (15, M^+), 566 (69), 531 (100), 425 (39), 281 (12), 147 (26), and 103 (17).

Band 3 was a single metabolite (VI, Table 1) which was identified as 3″,7-dihydroxy-CBD [m.s. fragments *m/e* 634 (17, M^+), 566 (42), 531 (100), 490 (2), 425 (38), 422 (9), and 268 (5)]. The ^{1}H-nmr gave: δ ($CDCl_3$) 6·25 (s, 2H, C-3′, C-5′), 5·86 (s, br, 1H, C-2), 4·66 and 4·56 (s, 1H, C-9), 4·12 (s, 2H, C-7), 3·54 (p, 1H, C-3″), 2·54 (m, 2H, C-1″), 1·67 (s, 3H, C-10), and 0·93 (t, 3H, C-5″).

T.l.c. showed the presence of two metabolites in band 4 (R_F = 0·47 and 0·38). The less polar metabolite (VII) was determined to be 1″,7-dihydroxy-CBD on the basis of *m/e* 634 (17, M^+), 566 (34), 531 (100),

Table 1. *Dioxygenated metabolites of CBD.*

Metabolite	Quantity (μg)	t.l.c.[a] (R_F)	g.l.c.[b] (min)	M^+	m.s. (*m/e*)[c] 100%	major distinguishing peaks
6,7–DiOH–CBD (III)	97	0·28	3·8	634	566	478, 337, 103
1″,7–DiOH–CBD (VII)	35[d]	0·46	3·0	634	531	509, 425, 159
3″,7–DiOH–CBD (VI)	318	0·29	5·6	634	531	490, 425, 268
4″,7–DiOH–CBD (IV)	980	0·24	6·1	634	531	425, 117
5″,7–DiOH–CBD (V)	60	0·18	7·2	634	531	425
2″,6–DiOH–CBD (X)	10	0·63	2·7	634	566	425, 145
3″,6β–DiOH–CBD (IX)	28	0·50	5·7	634	566	425, 268
4″,6β–DiOH–CBD (VIII)	60	0·37	6·0	634	566	425, 117
3″–OH–6–oxo–CBD (XI)	15	0·63	4·7	560	425	492, 416, 268
4″–OH–6–oxo–CBD (II)	60	0·50	5·1	560	425	492, 117

[a] Developed in 1% methanol—ether.
[b] Retention time of the silylated derivative on 2% SE-30 (250°).
[c] M.s. of silylated derivative at 20 eV.
[d] Calculated from g.l.c. data.

509 (17), 425 (38), 254 (12), and 159 (7). The more polar metabolite (VIII) was tentatively assigned the structure of 4″, 6β-dihydroxy-CBD as a result of m.s. fragments *m/e* 634 (4, M^+), 566 (100), 478 (3), 425 (23), and 117 (8). The β position was established (see Discussion) from the m.s. of the nonsilylated compound [*m/e* 346 (0), 328 (100), 273 (79), 256 (18), 247 (28), and 209 (48)].

Band 5 was composed of 1″,7-dihydroxy-CBD, 4″-hydroxy-6-oxo-CBD (traces), and a third metabolite (IX) which had an m.s. consistent with 3″,6β-dihydroxy-CBD [*m/e* 634 (4, M^+), 566 (100), 492 (7), 465 (5), 425 (30), 404 (4), and 268 (9)]. Specific fragments in the m.s. of the non-silylated compound were *m/e* 346 (0), 328 (75), 273 (63), 256 (100), 247 (68), and 209 (53). As expected, 3″,6β-dihydroxy-CBD was less polar than 4″,6β-dihydroxy-CBD on both t.l.c. and g.l.c. (Table 1).

Band 6 appeared as a single component on t.l.c., but on g.l.c. it was resolved into two metabolites (X and XI). Metabolite X (Rt = 2·7 min) was assigned the structure of 2″,6-dihydroxy-CBD based on the following m.s. fragments: *m/e* 634 (5, M^+), 566 (100), 425 (17), and 145 (63). Metabolite XI (Rt = 4·7 min) was found to be 3″-hydroxy-6-oxo-CBD. The distinguishing m.s. fragments were *m/e* 560 (28, M^+), 492 (82), 425 (100), 416 (44), and 268 (20). Further support was provided by the m.s. of the non-silylated compound by *m/e* 344 (22, M^+), 272 (46), 247 (100), 229 (72) and 175 (30).

Metabolites in band 7 were not identified.

DISCUSSION

CBD was converted by rat liver enzymes to ten dioxygenated metabolites which were categorized as follows: 6,7-dihydroxylation, 7-hydroxylation with side chain hydroxylation, 6-hydroxylation with side chain hydroxylation, and 6-keto formation accompanied by side chain hydroxylation. From the data summarized in Table 1, it was evident that the metabolic pattern was similar to that of the monohydroxylated CBD metabolites (Martin & others, 1976). Almost 90% of the dioxygenated material was present as a 7-hydroxy metabolite. Hydroxylation in the side chain occurred to the greatest extent at C-4″ and to a lesser degree at C-3″. Only trace amounts of the C-1″, -2″, or -5″ hydroxy metabolites were formed. As in the monohydroxy series, the polarity of the metabolites tended to increase as hydroxylation occurred nearer the end of the side chain.

The structural identification of dioxygenated metabolites was made possible by the comparison of their m.s. data with those of mono-oxygenated CBD metabolites (Martin & others, 1976). Fortunately, most metabolites were dioxygenated in such a way that the m.s. characteristics of each hydroxyl or ketone were still apparent (distinctive m.s. fragments summarized in Table 1). In the m.s. of the silylated dihydroxy compounds, 6-hydroxylation was distinguished by a base peak M^+-68 which resulted from a Retro-Diels-Alder (RDA) reaction. Budzikiewicz, Alpin & others (1965) showed that the RDA reaction in CBD involved the loss of C-4, 5, 8, 9 and 10 (see Fig. 2). The spatial orientation of the 6-hydroxy group was established by the presence of *m/e* 328 and 273 in the m.s. of the non-silylated metabolites. A 6α-hydroxy metabolite should have had *m/e* 346 (M^+) and a prominent *m/e* 278 (6α-hydroxy-CBD base peak + 16), while a 6β-hydroxy metabolite should have exhibited *m/e* 328 (M^+-18) and an abundant *m/e* 273 (6β-hydroxy-CBD base peak + 16) as described by Martin & others (1976). After silylation 6-oxo-CBD had a prominent M^+-68 (RDA) and a base peak *m/e* 337 which resulted from cleavage of the entire monoterpene moiety with the exception of C-3. Likewise, the 6-oxo metabolites had an abundant M^+-68 and a base peak *m/e* 425 (337 + side chain hydroxylation). Additional evidence was provided by the base peak M^+-97 which was common for both non-silylated 6-oxo-CBD and the 6-oxo metabolites.

The m.s. of silylated 7-hydroxy-CBD had a specific base peak M^+-103 as well as *m/e* 103 which was the same for the 7-hydroxylated-side chain hydroxy metabolites. Hydroxylation at C-7 could be distinguished from that at C-10 by the base peak (M^+-156 for silylated 10-hydroxy-CBD) as discussed by Martin & others (1976). In 6, 7-dihydroxy-CBD the base peak (*m/e* 566) indicated 6-hydroxylation (RDA), while *m/e* 103 specified 7-hydroxylation. In addition, the prominent *m/e* 337 showed that dihydroxylation had occurred only in the monoterpene moiety.

In the m.s. of the silylated compounds the distinguishing features of side chain hydroxylation were the presence of an abundant *m/e* 425 (described above) and the absence of *m/e* 337. Determination of the exact location of the hydroxy group depended upon the following fragments: *m/e* 509 (RDA coupled with the loss of $-C_4H_9$) and *m/e* 159 for 1″-hydroxy; *m/e* 145 $[(CH_3)_3\text{-}SiO = C_4H_8]^+$ for 2″-hydroxy; $M^+ - 144$, base peak—144, or *m/e* 268 for 3″-hydroxy; and *m/e* 117 $[(CH_3)_3SiO = CHCH_3]^+$ for 4″-hydroxy. 5″-Hydroxy exhibited a *m/e* 425 but was devoid of all fragments typical of the other side chain hydroxy compounds. A very intense base peak

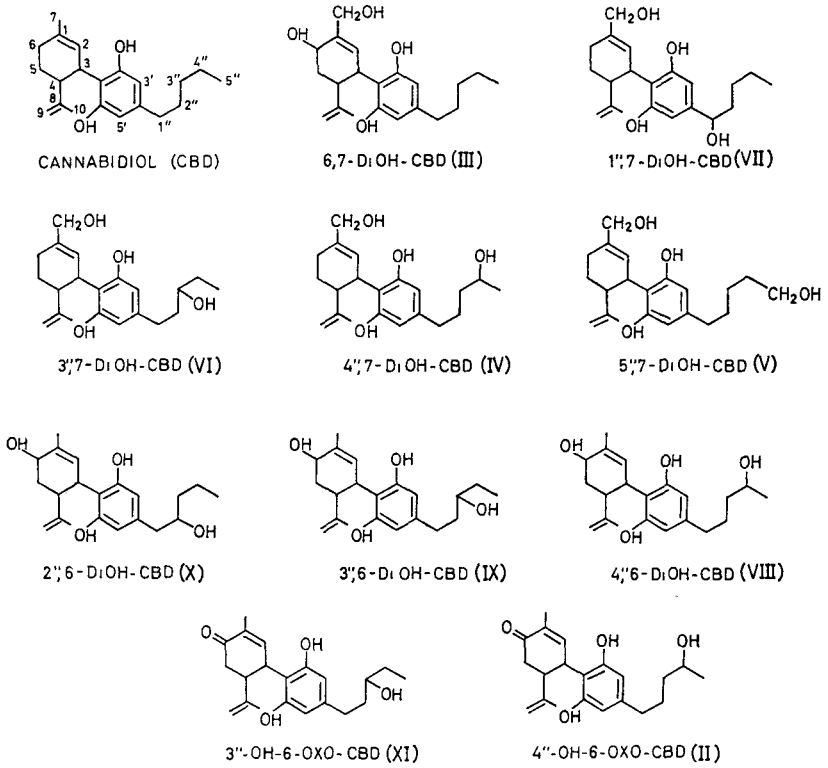

FIG. 2. Structures of dioxygenated CBD metabolites.

of the silylated 6-hydroxy compounds tended to reduce all other abundances and make differentiation between the side chain hydroxyls somewhat difficult. However, a strong *m/e* 145 was still evident in 2″,6-dihydroxy-CBD. The m.s. of non-silylated 3″,6β-dihydroxy-CBD, as well as 3″-hydroxy-6-oxo-CBD, provided additional evidence for 3″-hydroxylation, since a benzylic cleavage with proton transfer (M^+-72) was specific for 3″-hydroxy-CBD (Martin & others, 1976). The base peak in 3″6,β-dihydroxy-CBD resulted from $M^+-18-72$, and in 3″-hydroxy-6-oxo-CBD the prominent fragments *m/e* 272 and 175 arose from M^+-72 and base peak-72, respectively. 2″,6- and 3″,6β-dihydroxy-CBD were conclusively identified, but the structure of 4″,6β-dihydroxy-CBD could be assigned only on a tentative basis. The abundance of *m/e* 117 implied 4″-hydroxylation. Also, the chromatographic behaviour was consistent with that expected for 4″,6β-dihydroxy-CBD.

Since sufficient quantities of 3″,7- and 4″,7-dihydroxy-CBD were available, their structures could be confirmed by nmr. In the spectra of each compound the C-7 methyl proton singlet (normally at δ 1·82) was shifted downfield to δ 4·12 and was integrated as 2 protons. A similar spectrum has been reported for 7-hydroxy-CBD (Nilsson, Agurell & others, 1973). 3″-Hydroxylation was indicated by a pentet at δ 3·54, while 4″-hydroxylation was evident by the replacement of the C-5″ triplet at δ 0·87 with a doublet at δ 1·19.

Previously, dihydroxy metabolites have been reported for cannabinoids other than CBD. Wall, Brine & others (1970) identified 6,7-dihydroxy-Δ^1-THC. Later, Wall (1971) reported 5α,7- and 5β,7-dihydroxy-Δ^6-THC and the tentative identification of 2″,7-dihydroxycannabinol. It remains to be established whether the *in vitro* metabolism of these cannabis constituents is as diversified as that of CBD. Of equal significance is the determination of the pharmacological importance of these dioxygenated CBD metabolites.

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REFERENCES

AGURELL, S., GUSTAFSSON, B., GOSZTONYI, T., HEDMAN, K. & LEANDER, K. (1973). *Acta chem. scand.*, **27**, 1090–1091.

AGURELL, S., BINDER, M., FONSEKA, K., LINDGREN, J.-E., LEANDER, K., MARTIN, B., NILSSON, I. M., NORDQVIST, M., OHLSSON, A. & WIDMAN, M. (1976). Raven Press, in the press.

BAILEY, K. & TOFT, P. (1973). *Biochem. Pharmac.*, **22**, 2780–2783.

BUDZIKIEWICZ, H., ALPIN, R. T., LIGHTNER, D. A., DJERASSI, C., MECHOULAM, P. & GAONI, Y. (1965). *Tetrahedron*, **21**, 1881–1888.

CARLINI, E. A., LEITE, J. R., TANNHAUSER, M. & BERARDI, A. C. (1973). *J. Pharm. Pharmac.*, **25**, 664–665.

CHRISTENSEN, H. D., FREUDENTHAL, R. D., GIDLEY, J. D., ROSENFELD, R., BOEGLI, G., TESTINO, L., BRINE, D. R., PITT, C. G. & WALL, M. E. (1971). *Science*, **172**, 165–167.

IZQUIERDO, I. & TANNHAUSER, M. (1973). *J. Pharm. Pharmac.*, **25**, 916–917.

HOLLISTER, L. E. (1973). *Experientia*, **29**, 825–826.

JONES, G. & PERTWEE, R. G. (1972). *Br. J. Pharmac.*, **45**, 375–377.

JONES, G., WIDMAN, M., AGURELL, S. & LINDGREN, J. E. (1974). *Acta pharm. suecica*, **11**, 283–294.

KARNIOL, I. G. & CARLINI, E. A. (1972). *J. Pharm. Pharmac.*, **24**, 833–835.

KARNIOL, I. G., SHIRAKAWA, I., KASINSKI, N., PFEFERMAN, A. & CARLINI, E. A. (1974). *Eur. J. Pharmac.*, **28**, 172–177.

MARTIN, B., NORDQVIST, M., AGURELL, S., LINDGREN, J.-E., LEANDER, K. & BINDER, M. (1976). *J. Pharm. Pharmac.*, **28**, 275–279.

NILSSON, I., AGURELL, S., NILSSON, J. L. G., WIDMAN, M. & LEANDER, K. (1973). *Ibid.*, **25**, 486–487.

PEREZ-REYES, M., TIMMONS, M. C., DAVIS, K. H. & WALL, M. E. (1973). *Experientia*, **29**, 1368–1369.

TRUITT, E. B. (1971). *Pharmac. Rev.*, **23**, 273–278.

WALL, M. E., BRINE, D. R., BRINE, G. A., PITT, C. G., FREUDENTHAL, R. I. & CHRISTENSEN, H. D. (1970). *J. Am. chem. Soc.*, **92**, 3466–3468.

WALL, M. E. (1971). *Ann. N.Y. Acad. Sci.*, **191**, 23–37.

WIDMAN, M., NORDQVIST, M., AGURELL, S., LINDGREN, J.-E. & SANDBERG, F. (1974). *Biochem. Pharmac.*, **23**, 1163–1172.