Studies on Lysergic Acid Diethylamide and Related Compounds. Part 8.[1] Structural Identification of New Metabolites of Lysergic Acid Diethylamide obtained by Microbial Transformation using *Streptomyces roseochromogenes* †

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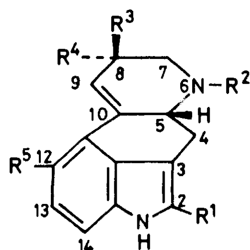
Microbial transformation of lysergic acid diethylamide (1) by *Streptomyces roseochromogenes* gave a known product, lysergic acid ethylamide (5), and two new ones. The structures of these new metabolites have been chemically established as lysergic acid ethylvinylamide (7) and lysergic acid ethyl-2-hydroxyethylamide (8).

SEVERAL reports have appeared on the metabolism of lysergic acid diethylamide (LSD) (1), a strong hallucinogenic agent. In 1957, Axelrod *et al.*[2] claimed formation of 2-hydroxylysergic acid diethylamide (2) by enzymatic systems of mammalian liver microsomes supplemented with NADPH and oxygen. In 1959, Boyd [3] showed that administration of LSD (1) to rats resulted in the biliary



(1) ; $R^1 = R^4 = R^5 = H, R^2 = Me, R^3 = CONEt_2$
(2) ; $R^1 = OH, R^2 = Me, R^4 = R^5 = H, R^3 = CONEt_2$
(3) ; $R^1 = R^4 = H, R^2 = Me, R^3 = CONEt_2, R^5 = OH$
(4) a ; $R^1 = R^3 = H, R^2 = Me, R^4 = CONEt_2, R^5 = OH$
b ; $R^1 = R^3 = R^5 = H, R^2 = Me, R^4 = CONEt_2$
(5) ; $R^1 = R^4 = R^5 = H, R^2 = Me, R^3 = CONHEt$
(6) ; $R^1 = R^4 = R^5 = H, R^2 = H, R^3 = CONEt_2$
(7) ; $R^1 = R^4 = R^5 = H, R^2 = Me, R^3 = CON(CH{=}CH_2)(Et)$
(8) ; $R^1 = R^4 = R^5 = H, R^2 = Me, R^3 = CON(CH_2CH_2OH)(Et)$
(10) ; $R^1 = R^4 = R^5 = H, R^2 = Me, R^3 = CO_2H$

excretion of two Ehrlich positive metabolites, and Slaytor *et al.*,[4] in 1962, suggested that these metabolites were the β-glucuronides of 12-hydroxylysergic acid diethylamide (3) and 12-hydroxyisolysergic acid diethylamide (4a). Later, however, Szara [5] cast doubt on their structures and claimed that hydroxylation could take place at C-13 of the lysergic acid skeleton.

Recently, two of us [6] (T. N. and Y. N.) re-examined the metabolism of LSD (1) at the subcellular level and isolated two metabolites, the structures of which were rigidly established as lysergic acid ethylamide [7] (LAE) (5) and norlysergic acid diethylamide [8] (norLSD) (6). At the time, we assumed that the metabolism of a foreign compound would proceed in an essentially similar manner in both mammals and micro-organisms, and the identification of metabolites produced by microbial transformation of LSD (1) would facilitate the further detailed studies on the metabolic pathway of LSD (1) in mammals. Then we [1] examined the transformation of LSD (1) with several *Streptomyces* species and found that *S. roseochromogenes* produced two new metabolites, lysergic acid ethylvinylamide (LEV) (7) and lysergic acid ethyl-2-hydroxyethylamide (LEO) (8), with a known metabolite, LAE (5), but *S. lavendulae* gave the known norLSD (6) as a sole metabolite. In this paper, we wish to describe the establishment of the structures of these new metabolites.

LEV (7) was isolated as labile colourless needles, m.p. 86—88 °C, which show $\nu_{max.}$ 1 660 and 1 625 cm^{-1} (CONC=C). In the mass spectrum, it shows a parent peak at *m/e* 321 corresponding to the molecular formula $C_{20}H_{23}N_3O$, where two hydrogen atoms have been removed from the molecule of LSD (1). The correctness of this formulation was confirmed by determination of the high-resolution mass spectrum of its parent ion. In an earlier paper, we [9] (T. N. and Y. N.) discussed the fragmentation of several derivatives of lysergic acid and proposed a general pathway which involved fragment ions, $M^+ - 43$ (CH_3–N=CH_2) [ion a: retro-Diels-Alder reaction at the ring D], M^+ — CONRR′ [ion b: removal of the side-chain at C-8], and M^+ — CONRR′ — 2H[ion c: aromatization of ion b at the ring D]. Appearance of the corresponding ions at *m/e* 278 (ion a), 223 (ion b), and 221 (ion c) in the mass spectrum of LEV (7) unequivocally indicates that the skeleton of lysergic acid is retained in LEV (7). Furthermore, the presence of a peak at *m/e* 295 corresponding to an ($M^+ - C_2H_2$) ion suggested that LEV (7) has a vinyl group on a nitrogen amide atom. In the n.m.r. spectrum, LEV (7) shows the presence of an ethylamide group [δ 1.22 (3 H, t, *J* 7 Hz, CH_3) and δ 3.77

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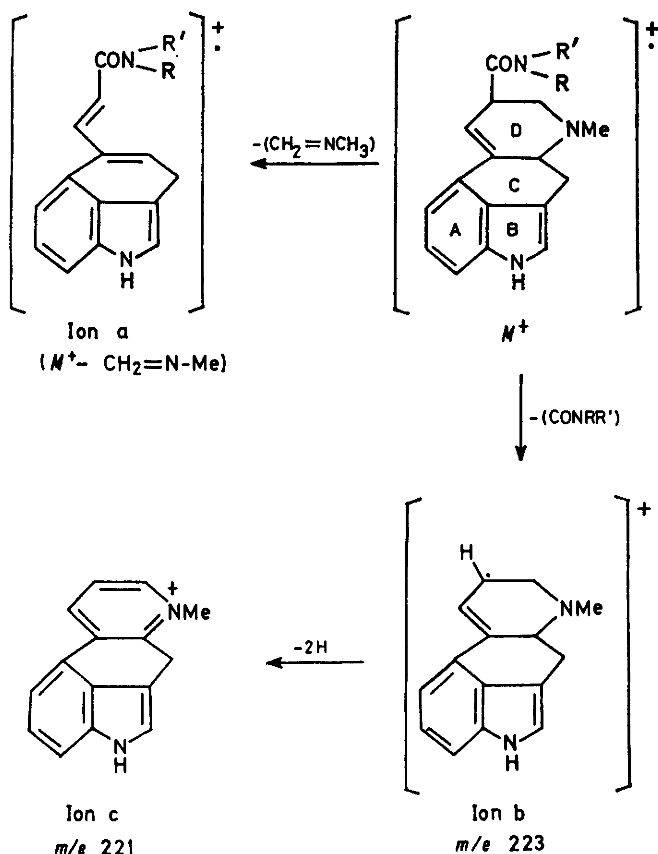
(2 H, q, J 7 Hz, CH_2N)] and a vinyl amide group [δ 4.45 (1 H, m, NCH=CH_AH$_B$), δ 4.60 (1 H, m, NCH=CH$_A$$H_B$), and δ 6.9—7.1 (1 H, m, NCH=CH$_A$H$_B$)]. This spectral evidence allowed us to assign the structure of lysergic acid ethylvinylamide (7) to this metabolite, except for the asymmetric centre of C-8.

In order to confirm the configuration of C-8, LEV (7) was hydrogenated over platinum black to give LSD (1) and dihydrolysergic acid diethylamide (I) [10] (9) in 75 and 10% yields respectively, demonstrating that LEV (7)

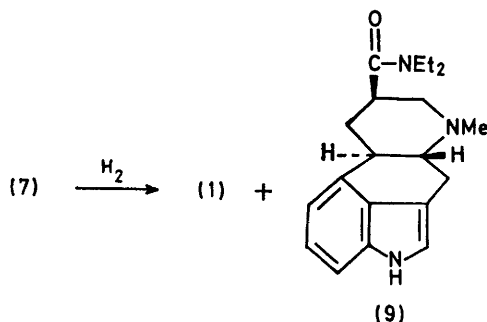


SCHEME 1

has the same configuration at C-8 as LSD (1). This evidence established rigidly the structure of LEV (7) as lysergic acid ethylvinylamide. It should be noted here that LEV (7) is so labile that treatment of it with either acid or base gives LAE (5) and all efforts to synthesize it from lysergic acid (10) itself failed.

Another new metabolite, LEO (8), was obtained as colourless needles, m.p. 108—109 °C, which show $\nu_{max.}$ 3 430 (OH) and 1 610 cm^{-1} (CON<). In the mass spectrum, it shows its parent peak at m/e 339 corresponding to the molecular formula $C_{20}H_{25}N_3O_2$. This elemental composition of LEO (8) was confirmed by examination of the high-resolution mass spectrum of its parent ion. LEO (8) could not be such a phenolic compound formed by oxidation of the skeleton of LSD (1) as reported by several research groups,[4,5] since the i.r. spectrum of its acetate (11) shows an alcoholic acetate band at $\nu_{max.}$ 1 740 cm^{-1}.

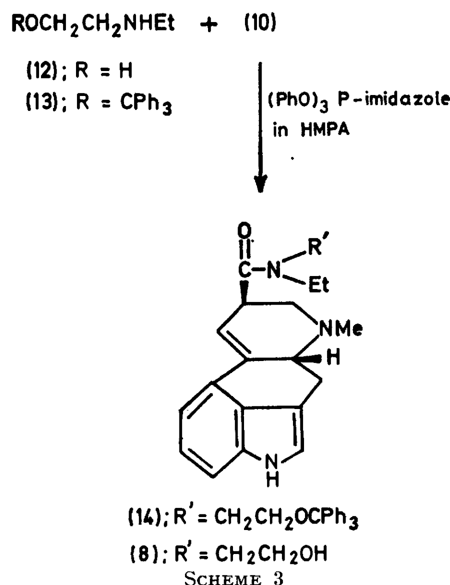
In the mass spectrum of LEO (8), three important fragment ions indicating the presence of a lysergic acid moiety in the molecule were observed at m/e 296 (ion a), 223 (ion b), and 221 (ion c). This fact strongly suggests that LEO (8) is a product hydroxylated on the amide



SCHEME 2

chain of the starting LSD (1). In the n.m.r. spectrum, LEO (8) shows the signals due to one ethyl group [δ 1.22 (3 H, t, J 7 Hz, CH_3) and δ 3.49 (2 H, q, J 7 Hz, CH_2N)] and a new A_2X_2 type of signal, attributable to an NCH_2-CH_2O group [δ 3.56 (2 H, t, J 5 Hz, NCH_2), and δ 3.80 (2 H, t, J 5 Hz, CH_2O)]. On the basis of these results we hypothesized that LEO (8) would be lysergic acid ethyl-2-hydroxyethylamide. We therefore undertook synthesis of this compound from lysergic acid (10).

N-Ethylethanolamine (12) was treated with trityl chloride to give ethyl-2-trityloxyethylamine (13) as an oily product in 67% yield. Lysergic acid (10) was



SCHEME 3

treated with triphenyl phosphite–imidazole complex in hexamethylphosphoric triamide (HMPA), followed by ethyl-2-trityloxyethylamine (13) to give lysergic acid ethyl-2-trityloxyethylamide (14) as an amorphous product in 72% yield. This reaction [7] retains the optical centre of lysergic acid at C-8.

Treatment of the trityl amide (14) with acetic acid afforded lysergic acid ethyl-2-hydroxyethylamide (LEO) (8) in 95% yield. The synthetic material was identical with the sample of LEO (8) obtained by microbial transformation.

Slaytor *et al.*[4] reported formation of an epimerized product in the mammalian metabolism of LSD (1) but the confirmatory evidence for retention of the optical centre at C-8 in LEO (8) came from the observation of its o.r.d. curve. In a previous paper,[7] we (Y. N. and T. N.) revealed that, in the o.r.d. spectrum, the amide derivatives of lysergic acid (10) having an amide group at the C(8)-β pseudoequatorial position and those of isolysergic acid (4b) having it at the C(8)-α pseudoaxial showed a peak at *ca.* 335 nm. The values of molecular rotation of the former correspond to $+8°$ for $+13° \times 10^3$, while those of the latter to $+17°$ for $+20° \times 10^3$. In LEO (8), molecular rotation of the peak observed at 334 nm has a value $+10.8° \times 10^3$, indicating that the amide group should be located at C(8)-β.

It is of importance that the isolation of LEV (7) is apparently the first indication of the presence of a metabolic pathway in which the alkyl chain of an amide group is oxidized to a vinyl amide group. Furthermore, compound (7) was recently also found in the mixture of mammalian metabolites of LSD (1) in a subcellular experiment [11] using the LEV (17) obtained in this work as a reference sample. The fact that three out of four microbial metabolites were identical with mammalian metabolites suggests that the metabolism of a foreign compound even in different species of organism proceeds along a similar pathway. In view of the fact that microbial transformation can easily afford sufficient quantities of metabolites for structural establishment, suggests that these experiments are valuable for the study of foreign compound metabolism in mammals either as a model or as an excellent auxiliary procedure.

EXPERIMENTAL

M.p.s were taken on a hot-stage apparatus. I.r., u.v., and mass spectra were measured with JASCO DS-701G, Hitachi EPS-3T, and JEOL JMS-01SG spectrometers, respectively. ^{1}H N.m.r. spectra were measured with a Hitachi R-22 (90 MHz) spectrometer for solutions in deuteriochloroform with tetramethylsilane as internal standard. Confirmation of purity by t.l.c. on silica gel was performed on each product with three solvent systems: (i) chloroform–methanol [4 : 1 (v/v)] (solv. A), (ii) chloroform–acetone [1 : 4 (v/v)] (solv. B), (iii) methanol–chloroform–n-hexane [1 : 4 : 2 (v/v/v)] (solv. C). T.l.c. detection was effected with u.v. light (365 nm) and/or Ehrlich reagent (*p*-dimethylaminobenzaldehyde in alcoholic HCl). The starting materials [7] used in this study were prepared in our laboratory from D-lysergic acid purchased from the Sigma Co. Ltd.

Strain.—*S. roseochromogenes* (IMF 1081) stored at the Research Institute for Chemobiodynamics, Chiba University, was used.

Microbial Transformation of LSD (1).—The mycelium and/or spore of *S. roseochromogenes* from stock culture was suspended in 100 ml of soybean medium [soybean meal, 30 g; soluble starch, 20 g; NaCl, 1 g; K_2HPO_4, 1 g; city water, 1 000 ml; pH adjusted to 7.2 with 1N-NaOH] and preincubated at 28 °C on a reciprocating shaker at 60 r.p.m. for 48 h. The preincubated culture (3 ml) was inoculated in 500-ml Sakaguchi flasks containing soybean medium (100 ml) and incubated on a reciprocating shaker at 28 °C and 60 r.p.m. At 48 h after inoculation, a solution of LSD (1) in methanol (10 mg/ml) was added until its concentration reached to 0.1 mg/ml of medium (0.01%). Incubation was continued until conversion of LSD (1) reached a maximum [96 to 120 h after addition of LSD (1)].

The whole culture was added to three times its volume of ethanol and filtered or centrifuged. The precipitates were well washed with ethanol. The washings were combined with the filtrate and evaporated to 1/5 volume *in vacuo*. The concentrated solution (pH 8) was extracted with ethyl acetate. The combined extracts were evaporated to dryness *in vacuo*. The residue was dissolved in benzene and extracted with 1% tartaric acid. The acid layer was basified to pH 8.0 with sodium hydrogencarbonate and extracted with ethyl acetate. The extract was evaporated to dryness *in vacuo*.

The extract was separated into individual components by column chromatography using a 65 × 3 cm neutral alumina column [Merck, Brockmann (activity II—III), No. 1097] for 100 mg of the extract, and benzene–acetone, chloroform, ethyl acetate, and methanol as eluting solvents in order of elution. The metabolites were eluted in the order LEV (7) [with benzene–acetone (10 : 1) (v/v)], the recovered LSD (1) [with benzene–acetone (9 : 1) (v/v)], LAE (5) [with benzene–acetone (9 : 1 or 7 : 1) (v/v)], and LEO (8) [with chloroform]. Each fraction was purified by preparative t.l.c. to give LEV (7), the recovered LSD (1), LAE (5), and LEO (8) in 1.0, 28, 22, and 10% yields, respectively.

Lysergic Acid Ethylvinylamide (*LEV*) (7).—Recrystallization of the metabolite having R_F values 0.92 (solv. A), 0.60 (solv. B), and 0.61 (solv. C) on t.l.c. from benzene–n-hexane gave colourless needles, m.p. 86—88 °C; $\nu_{max.}$(KBr) 1 660 (C=C) and 1 625 cm^{-1} (amide); $\lambda_{max.}$(EtOH) 243 (log ε 4.29) and 313 nm (3.94); o.r.d. ($c = 1.72 \times 10^{-3}$%, MeOH) $[\phi]^{22°}$ +390 (500 nm), +1 640 (400), +9 770 (peak, 335), 0 (318), and −12 100° (300); δ 1.22 (3 H, t, *J* 7 Hz, CH_3), 2.59 (3 H, s, NCH_3), 2.7—4.2 (6 H, m, aliphatic H), 3.77 (2 H, q, *J* 7 Hz, NCH_2), 4.45 (1 H, m, H_A of $CH=CH_AH_B$), 4.60 (1 H, m, H_B of $CH=CH_AH_B$), 6.39 (1 H, s, 9-H), 6.9—7.1 (1 H, m, NCH=C), 6.91 (1 H, s, 2-H), 7.1—7.3 (3 H, m, ArH), and 8.03 (1 H, s, NH); *m/e* 321 (M^+, 100%), 295 ($M^+ - C_2H_2$, 33), 278 ($M^+ - CH_2{=}NCH_3$, 12), 221 ($M^+ - CON(C_2H_5)CH{=}CH_2 - 2$ H, 90) (Found M^+, 321.1841. $C_{20}H_{23}N_3O$ requires *M*, 321.1838).

Recovered Lysergic Acid Diethylamide (*LSD*) (1).—The product having R_F values 0.87 (solv. A), 0.27 (solv. B), and 0.49 (solv. C) on t.l.c., after recrystallization from benzene gave colourless prisms, m.p. 82—84 °C. This material was identical with LSD (1).

Lysergic Acid Ethylamide (*LAE*) (5).—The product having R_F values 0.74 (solv. A), 0.21 (solv. B), and 0.40 (solv. C) on t.l.c. was obtained as an oil. Treatment of this oily substance with an equivalent amount of D-tartaric acid afforded colourless prisms, m.p. 149—150 °C. This tartrate was identical with that of authentic LAE (5).[6,7]

Lysergic Acid Ethyl-2-hydroxyethylamide (*LEO*) (8).—Recrystallization of the product having R_F values 0.64 (solv. A), 0.11 (solv. B), and 0.25 (solv. C) on t.l.c., crystallized from ethyl acetate as colourless needles, m.p. 108—109

°C; $\nu_{max.}$(KBr) 3 430 (hydroxy) and 1 610 cm^{-1} (amide); $\lambda_{max.}$(EtOH) 243 (log ε 4.32) and 312 nm (3.96); o.r.d. (c = 2.50 × 10^{-3}%, MeOH) $[\phi]^{22°}$ +450 (500 nm), +1 770 (400), +10 800 (peak, 334), 0 (320) and −10 200° (300); δ 1.22 (3 H, t, J 7 Hz, CH_3), 2.5—4.0 (6 H, m, aliphatic H), 2.55 (3 H, s, NCH_3), 3.49 (2 H, q, J 7 Hz, NCH_2), 3.56 (2 H, t, J 5 Hz, NCH_2), 3.80 (2 H, t, J 5 Hz, OCH_2), 6.35 (1 H, d, 9-H), 6.87 (1 H, s, 2-H), 7.1—7.3 (3 H, m, ArH), and 8.25 (1 H, broad s, NH); m/e 339 (M^+, 100%), 309 (M^+ − CH_2O, 19), 296 (M^+ − CH_2=NCH_3, 8.5), 221 [M^+ − CON-(C_2H_5)CH_2CH_2OH − 2 H, 85] (Found: M^+ 339.1952. $C_{20}H_{25}N_3O_2$ requires M, 339.1947).

Catalytic Hydrogenation of LEV (7).—A solution of LEV (7) (8 mg) in ethyl acetate was hydrogenated over Pt black (20 mg) at atmospheric pressure and room temperature for 2 h. After removal of the catalyst by filtration, the filtrate was evaporated to dryness under reduced pressure. Preparative t.l.c. of the oily residue on silica gel using a mixed solvent (solv. A) gave two pure components. The main product (6 mg), corresponding to R_F 0.87 on t.l.c. (solv. A), was identical with LSD (1) and the minor product (0.8 mg), corresponding to R_F 0.78, with dihydrolysergic acid diethylamide (I) (9).[10b]

Lysergic Acid 2-Acetoxyethylethylamide (*AcLEO*) (11).—A solution of LEO (8) (10 mg) in benzene (20 ml) containing pyridine (1 ml) and acetic anhydride (1 ml) was warmed at 32 °C for 4 h; the reaction mixture was then extracted with 1% tartaric acid. The acid layer was basified to pH 10 with sodium hydrogencarbonate and then extracted with chloroform. The chloroform solution was dried (sodium sulphate) and evaporated to dryness under reduced pressure. The residue was purified by preparative t.l.c. using the mixed solvent (solv. C), to give a colourless oil (8 mg); $\nu_{max.}$($CHCl_3$) 1 740 (ester) and 1 615 cm^{-1} (amide); m/e 381 (M^+, 100%), 338 (M^+ − $COCH_3$, 75) (Found: M^+ 381.2075. $C_{22}H_{27}N_3O_3$ requires M, 381.2052).

Ethyl-2-trityloxyethylamine (13).—To a solution of *N*-ethylethanolamine (0.89 ml) was added sodium hydride (0.50 g; 57% suspended in mineral oil). Then trityl chloride (3.3 g) was added and the mixture stirred at room temperature for 2 h. After the precipitate produced during the reaction had been removed by filtration, the filtrate was evaporated to dryness under reduced pressure. The oily residue was dissolved in cyclohexane and chromatographed on silica gel (Mallinckrodt). After rejection of the initial cyclohexane eluate the fraction eluted with the mixed solvent, cyclohexane–ethyl acetate [9 : 1 (v/v)], gave a yellow oil (2.2 g); δ 1.05 (3 H, t, J 7 Hz, CH_3), 2.9—3.1 (1 H, broad s, NH), 2.43 (2 H, q, J 7 Hz, NCH_2), 2.56 (2 H, t, J 7 Hz, NCH_2), 3.78 (2 H, t, J 7 Hz, CH_2O), and 7.0—7.6 (15 H, m, ArH).

Lysergic Acid Ethyl-2-trityloxyethylamide (14).—The complex of triphenyl phosphite (0.93 ml) and imidazole (204 mg) in acetonitrile was added to a solution of LSD (1) (268 mg) in HMPA (3 ml) at room temperature. After addition of a solution of ethyl-2-trityloxyethylamine (13) (500 mg), the mixture was set aside at room temperature overnight; it was then diluted with a large quantity of benzene and extracted with 1% tartaric acid solution. The acid solution was made alkaline with saturated sodium hydrogencarbonate solution and extracted with benzene. The organic layer was dried (sodium sulphate) and evaporated to dryness under reduced pressure. The residue was chromatographed on alumina using benzene–acetone [9 : 1 (v/v)] as solvent to give an amorphous substance (418 mg); m/e 581 (M^+, 36%), 338 (M^+ − Ph_3C, 33), 243 (Ph_3C^+, 100), 221 [M^+ −CON(C_2H_5) − CH_2CH_2OTr − 2 H, 65].

Synthetic LEO (8).—A solution of the above trityloxyamide derivative (14) in 20% acetic acid solution (10 ml) was warmed at 80 °C for 30 min, made alkaline with saturated sodium hydrogen carbonate solution, and extracted with chloroform. The chloroform solution was dried (sodium sulphate) and evaporated to dryness under reduced pressure. Chromatography of the residue on alumina using benzene–acetone [9 : 1 (v/v)] (fraction 1) and ethyl acetate (fraction 2) as eluant gave colourless needles (230 mg) in fraction 2, m.p. 108—109 °C, which were recrystallized from ethyl acetate.

This material was identical with the sample of LEO (8) obtained by microbial transformation.

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