Biotransformation of Phorbol by Human Intestinal Bacteria

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Received June 26, 2001; accepted October 23, 2001

Anaerobic incubation of phorbol (1) from *Croton tiglium* with human intestinal bacteria afforded five metabolites: isophorbol (2), deoxyphorbol (3), 4β,9α,20-trihydroxy-13,15-seco-1,6,15-tigliatriene-3,13-dione (4), 4β,9α,20-trihydroxy-15,16,17-trinor-1,6-tigliadiene-3,13-dione (5) and 4β,9α,20-trihydroxy-14(13→12)-abeo-12α*H*-1,6-tigliadiene-3,13-dione (6). All these metabolites (2—6) were identified and characterized by spectroscopic means, including two-dimensional (2D)-NMR. Nine defined strains from the human intestine showed an ability to transform 1 to these metabolites.

Key words phorbol; isophorbol; biotransformation; human intestinal bacteria

Phorbol (**1**) is a novel tetracyclic polyol which was originally isolated as its esterified form from croton oil.[1)] As one of the hydrolyzates of the oil, **1** was isolated and its absolute configuration was determined by X-ray analysis.[2)] A wide variety of biochemical responses elicited by phorbol esters provided new opportunities for research on treatments for cancer, inflammation, cardiovascular diseases, memory development and acquired immunodeficiency syndrome (AIDS).[3—5)] The increased lipid solubility of phorbol esters over that of **1** itself may allow penetration of membrane structures of the sites of action without change in the configuration of the molecule or the location of its functional groups. Although many biological activities of phorbol esters are mediated through their direct interaction and the activation of protein kinase C (PKC), there are some phorbol esters that are active without being tumor promoters, such as ostodin and prostratin.[6,7)] Recently, Hattori *et al.* reported that 12-*O*-acetylphorbol 13-decanoate potently inhibited the cytopathic effects of human immunodeficiency virus type 1 without appreciable activation of protein kinase C.[8,9)] Phorbol (**1**) has been synthesized,[10)] and the total synthetic approach can be modified in ways which might lead to biologically improved versions of the molecule. Microbial transformation studies have been conducted successfully as model systems to predict metabolic pathways in humans or to increase the efficiency of drugs by metabolic activation. Heretofore, the metabolic studies of **1** involving human intestinal flora have not yet been reported. In the present paper, we now wish to report the isolation and structural determination of five metabolites obtained after anaerobic incubation of **1** with a faecal bacterial mixture from a human subject, followed by screening of defined bacteria capable of transforming **1** to its respective metabolites.

Experimental

Instruments Optical rotations were measured in MeOH or dioxane using a JASCO DIP-360 automatic polarimeter at 25 °C. Infrared (IR) spectra were measured with a JASCO FT/IR-230 infrared spectrophotometer. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were measured with a JEOL-JNM-GX 400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) or a Varian Unity 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometer, and all chemical shifts are given in δ ppm relative to tetramethylsilane (TMS). ^{1}H–^{1}H-correlated spectroscopy (COSY), ^{1}H-detected multiple quantum coherence (HMQC) and heteronuclear multiple-bond coherence (HMBC) experiments were performed with the usual pulse sequence, and data processing was obtained with standard Varian software. Electron impact (EI) mass spectra were measured with a JEOL JMS-AX 505 spectrometer at an ionization voltage of 70 eV. A Tabai anaerobic incubator, EAN-140, was used for anaerobic incubation. Densitometric profiles were recorded on a Shimadzu CS-910 dual wavelength thin layer chromatoscanner (TLC scanner).

Chromatography Thin layer chromatography was carried out on precoated Silica gel 60 F_{254} plates and reversed phase RP-18 F_{254} S plates (0.25 mm thickness, Merck, Darmstadt, Germany), and spots were detected under UV light or after spraying with anisaldehyde–H_2SO_4 reagent followed by heating. Silica gel 60 (70—230 mesh, Merck, Darmstadt, Germany) was used for column chromatography. Medium pressure liquid chromatography (MPLC) was carried out over a LiChroprep RP-18 column (Merck, size A). Analytical high pressure liquid chromatography was performed on a CCPM-II (Tohso, Tokyo, Japan) equipped with a Tosho UV-8020 spectrometer and a Shimadzu C-R 8A chromatopac (Shimadzu Co., Ltd., Kyoto, Japan) utilizing a Tosho ODS-80Ts column [4.6 (i.d.)×150 m]. The flow rate was kept at 1.0 ml/min and the peaks were monitored at 254 nm using methanol–acetonitrile–water (2 : 1 : 5) as a solvent system.

Chemicals General anaerobic medium (GAM) broth was purchased from Nissui Seiyaku Co., Ltd. (Tokyo, Japan). Phorbol (**1**) and isophorbol (**2**) were isolated and purified from croton oil according to the method of Mishra *et al.*[11)] [its purity (approx. 100%) was determined by HPLC under the above mentioned conditions], where croton oil (150 ml) was stirred under an atmosphere of argon for 20 h with a solution of $Ba(OH)_2 \cdot 8H_2O$ (2.2% in methanol, 1000 ml) at room temperature. The formed precipitates were removed from the reaction mixture by filtration. The filtrate was dried *in vacuo* and the residue was suspended in water (650 ml) and shaken with ether (300 ml×2). The aqueous layer was acidified by 2 N H_2SO_4 to pH 5, and the mixture was kept at room temperature for 1 h. The precipitates were removed and the filtrate was neutralized by 2 N NaOH to pH 7, then the solvent was evaporated *in vacuo*. Hot ethanol (700 ml) was added to the residue, and the precipitates formed were removed by filtration. The filtrate was then evaporated *in vacuo* to give a crude phorbol fraction (26 g). Column chromatography of the fraction on silica gel using $CHCl_3$ with increasing amounts of MeOH (9.5 : 0.5—8 : 2) yielded phorbol (**1**, 1.8 g) and isophorbol (**2**, 2.7 g).

Microorganisms Strains of human intestinal bacteria were provided by Professor T. Mitsuoka, Tokyo University.

Preparation of a Human Intestinal Bacterial (HIB) Mixture Fresh feces (5 g) obtained from a healthy subject was mixed with 50 mM K–phosphate buffer (50 ml, pH 7.3), and the sediments were removed by decantation. The suspension was made up to 100 ml with addition of the same buffer, and was used as an HIB mixture in this experiment.

Transformation of 1 by an HIB Mixture A pre-cultured bacterial suspension (300 ml) was added to GAM broth (3000 ml), and the mixture was cultured for 24 h at 37 °C under anaerobic conditions. The culture was centrifuged at 8000×***g*** for 10 min. The pellets were washed with saline and suspended in GAM broth (1000 ml). Phorbol (**1**, 1 g in 2 ml MeOH) was added to the bacterial suspension, and the mixture was anaerobically incubated at 37 °C for 3 d. The reaction mixture was extracted with ethyl acetate (500 ml×4). The residue (2.4 g) after the evaporation of ethyl acetate *in vacuo* was chromatographed over silica gel eluted with $CHCl_3$–MeOH (9 : 1) to give fractions A (150 mg) and B (315 mg). Fraction A was separated by MPLC using a LiChroprep RP-18 column with MeOH–H_2O (4 : 6) as a mo-

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bile phase to yield compounds **2** (9 mg) and **3** (5 mg). Fraction B was also separated by MPLC with RP-18 (MeOH–H_2O, 3 : 7) to afford compounds **4** (28 mg), **5** (16 mg) and **6** (4 mg).

Compound **2**: White amorphous material. IR (KBr) ν_{max} cm^{-1}: 3450 (OH), 1698 (5-membered ring α-unsaturated C=O) and 1640 (C=C). EI-MS *m/z* (rel. int.): 364 [M^+] (25), 346 [M^+-H_2O] (50), 328 [M^+-2H_2O] (75), 310 [M^+-3H_2O] (100). ^{1}H-NMR (CD_3OD, 400 MHz) δ: 0.56 (1H, d, *J*=5.6 Hz, H-14), 1.14 (3H, s, H-17), 1.21 (3H, d, *J*=6.4 Hz, H-18), 1.24 (3H, s, H-16), 1.57 (1H, m, H-11), 1.71 (3H, m, H-19), 1.78 (1H, m, H-8), 2.14 (1H, d, *J*=14.2 Hz, H_a-5), 3.2 (1H, t, *J*=2.44 Hz, H-10), 3.41 (1H, d, *J*=14.0 Hz, H_b-5), 3.78 (2H, ABq, *J*=14.0 Hz, H-20), 4.03 (1H, d, *J*=10.2 Hz, H-12), 5.2 (1H, br s, H-7) and 7.28 (1H, m, H-1). ^{13}C-NMR see Table 1.

Compound **3**: White amorphous material. IR (KBr) ν_{max} cm^{-1}: 3350 (OH), 1695 (5-membered ring α-unsaturated C=O) and 1630 (C=C). EI-MS *m/z* (rel. int.): 364 [M^+] (65), 330 [M^+-H_2O] (100). ^{1}H-NMR (CD_3OD, 400 MHz) δ: 0.52 (1H, d, *J*=5.1 Hz, H-14), 1.12 (3H, s, H-17), 1.22 (3H, d, *J*=6.1 Hz, H-18), 1.24 (3H, s, H-16), 1.56 (1H, m, H-11), 1.71 (3H, m, H-19), 1.84 (1H, br t, H-8), 2.23 (1H, dd, *J*=18.2, 4.6 Hz, H_a-5), 2.69 (1H, m, H-4), 3.17 (1H, dd, *J*=15.1, 2.4 Hz, H_b-5), 3.5 (1H, m, H-10), 3.83 (2H, ABq, *J*=14.6 Hz, H-20), 4.02 (1H, d, *J*=10.5 Hz, H-12), 5.13 (1H, br s, H-7) and 7.28 (1H, br s, H-1). ^{13}C-NMR see Table 1.

Compound **4**: White amorphous material. $[\alpha]_D$ +182° [*c*=0.12, MeOH; *cf.* $[\alpha]_D^{23}$ +186° (*c*=0.41, MeOH)[15]], IR (KBr) ν_{max} cm^{-1}: 3400 (OH), 1710 (6-membered ring C=O), 1695 (5-membered ring α-unsaturated C=O) and 1640 (C=C). EI-MS *m/z* (rel. int.): 346 [M^+] (42), 328 [M^+-H_2O] (100), 300 [M^+-H_2O-CO] (39). ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 1.02 (3H, d, *J*=7.0 Hz, H-18), 1.64 (3H, s, H-17), 1.81 (3H, m, H-19), 2.31 (2H, dd, *J*=20.0, 5.5 Hz, H-12), 2.5 (2H, m, H-5), 2.68 (1H, m, H-11), 3.13 (1H, t, *J*=2.5 Hz, H-10), 3.39 (1H, d, *J*=13.0 Hz, H-14), 3.71 (1H, m, H-8), 4.0 (2H, q, *J*=13.5 Hz, H-20), 4.82 (1H, br s, H_a-16), 5.03 (1H, t, *J*=1.5 Hz, H_b-16), 5.48 (1H, dd, *J*=5.5, 1.0 Hz, H-7) and 7.56 (1H, m, H-1). ^{13}C-NMR see Table 1.

Compound **5**: White amorphous material. $[\alpha]_D$ +109° [*c*=0.15, dioxan; *cf.* $[\alpha]_D^{20}$ +107° (*c*=1%, dioxan)[16]], IR (KBr) ν_{max} cm^{-1}: 3450 (OH), 1709 (6-membered ring C=O), 1695 (5-membered ring α-unsaturated C=O) and 1630 (C=C). EI-MS *m/z* (rel. int.): 306 [M^+] (35), 289 [M^+-OH] (18), 276 [M^+-CH_3] (68). ^{1}H-NMR (CD_3OD, 500 MHz) δ: 0.92 (3H, d, *J*=7.0 Hz, H-18), 1.76 (3H, m, H-19), 2.06 (1H, ddd, *J*=18.6, 8.55, 5.8 Hz, H_a-12), 2.18 (1H, ddd, *J*=18.6, 7.95, 5.2 Hz, H_b-14), 2.41 (1H, m, H_a-5), 2.50 (1H, m, H_b-12), 2.52 (1H, m, H_b-5), 2.59 (1H, m, H-11), 2.84 (1H, t, *J*=15.0 Hz, H_b-14) 3.16 (1H, m, H-10), 3.6 (1H, m, H-8), 3.9 (2H, s, H-20), 5.34 (1H, m, H-7) and 7.61 (1H, m, H-1). ^{13}C-NMR see Table 1.

Compound **6**: White amorphous material. $[\alpha]_D$ −13.2° [(*c*=0.18, MeOH); *cf.* $[\alpha]_D$ −17.7° (MeOH)[15]], IR (KBr) ν_{max} cm^{-1}: 3450 (OH), 1765 (4-membered ring C=O), 1705 (5-membered ring α-unsaturated C=O) and 1635 (C=C). EI-MS *m/z* (rel. int.): 346 [M^+] (24), 300 [M^+-H_2O-CO] (55), 285 [M^+-CH_3] (14). ^{1}H-NMR (CD_3OD, 500 MHz) δ: 0.96 (3H, d, *J*=7.4 Hz, H-18), 1.22 (3H, s, H-16), 1.23 (3H, s, H-17), 1.76 (3H, m, H-19), 1.95 (1H, m, H_a-5), 2.45 (1H, m, H-14), 2.7 (1H, m, H_b-5), 2.87 (1H, m, H-11), 3.12 (1H, t, *J*= 3.0 Hz, H-10), 3.86 (1H, m, H-12), .3.93 (1H, m, H-8), 4.59 (2H, s, H-20), 5.45 (1H, d, *J*= 6.0 Hz, H-7) and 7.57 (1H, br s, H-1). ^{13}C-NMR see Table 1.

Time Course of the Metabolism of Phorbol (1) by an HIB Mixture from Human Feces To a suspension of the precultured bacterial mixture (3 ml) in GAM broth (10 ml), phorbol (**1**, 10 mg) was added, and the mixture was anaerobically incubated at 37 °C. A portion (1 ml) of it was taken each day for 4 d and extracted with ethyl acetate (1 ml). An aliquot (10 ml) of the ethyl acetate extract was applied to a TLC plate (Merk Kieselgel F_{245}, 0.25 mm thickness), and the plate was developed with $CHCl_3$–MeOH (9 : 1) and analyzed with a TLC scanner at 260 nm. The amounts of phorbol and its metabolites were determined using calibration lines prepared with authentic samples.

Screening of Bacterial Strains for Their Abilities to Metabolize Phorbol (1) Each precultured bacterial suspension (0.2 ml) was added to GAM broth (10 ml) and cultured for 24 h at 37 °C under anaerobic conditions. A portion (3 ml) of each culture was diluted to 10 ml with the same medium and incubated for another 24 h. Phorbol (**1**, 10 mg) was added to each culture, and the mixture was incubated for 3 d at 37 °C under anaerobic conditions. The incubation mixture was extracted with EtOAc (10 ml×3). The extract was evaporated *in vacuo* to afford a residue. The residue was dissolved in MeOH (1 ml) and analyzed quantitatively by TLC-densitometry.

Results and Discussion

By anaerobic incubation with a fecal bacterial mixture from humans for 3 d, phorbol (**1**) was transformed to various metabolites, which were then extracted with ethyl acetate and chromatographed on a silica gel column followed by MPLC (RP-18) to give 5 compounds (**2**—**6**) (Chart 1). The structures of these metabolites were determined by various spectroscopic means, including IR, NMR and MS spectroscopies.

Compounds **2** and **3** were identified to be isophorbol and deoxyphorbol, respectively, by comparing their IR, MS, ^{1}H-NMR (see Experimental part) and ^{13}C-NMR spectra (Table 1), as well as *Rf* values on TLC in various solvent systems with those of authentic samples.[12—14)]

The structure of **4** was determined by careful inspection of its spectroscopic data. The IR spectrum exhibited an absorption band at 1710 cm^{-1}, characteristic of a 6-membered ring ketone. The ^{1}H-NMR spectrum, with the aid of ^{1}H–^{1}H COSY, gave signals similar to those of **1**, except that two methyls (δ_H 1.1 and 1.21; H_3-16 and H_3-17 respectively) attached to a cyclopropane ring in **1** disappeared, but an isopropenyl group (vinyl: δ_H 4.82 and 5.03; methyl: δ_H 1.64) was assigned. In the EI-MS spectrum, the molecular ion peak was assigned at *m/z* 346 $[M]^+$ with 18 mass units less than that of **1**, suggesting that **4** is a dehydrated compound of **1**. Dehydroxylation at C-12 was indicated by an upfield shift of H-12 (δ_H 4.0 and 2.31 in **1** and **4**, respectively) along with a change in integration from one to two protons. Moreover, the presence of a carbonyl group at C-13 lead to a downfield shift of H-14 (δ_H 0.68 and 3.39 in **1** and **4**, respectively). The structure of **4** was further confirmed by analysis of the ^{13}C-NMR spectrum, in combination with distortionless enhancement by polarization (DEPT), HMQC and HMBC experiments, which indicated a pattern similar in part to that of **1**, except for signals due to one carbonyl carbon (δ_C 208.7, C-13), one olefinic carbon (δ_C 116.4, C-16) and a quaternary carbon (δ_C 141.1, C-15). The downfield shift of C-14 (δ_C 57.7) in **4** relative to that of **1** (δ_C 37.5) may be attributed to the desheilding effect of a carbonyl group at C-13. On the other hand, the dramatic upfield shift of the C-12 signal (δ_C 46.3) compared with that of **1** (δ_C 82.0) can be explained by dehydroxylation at C-12, which subsequently lead to the upfield shift of a C-11 signal (δ_C 37.8 and 46.1 in **4** and **1**, respectively). The chemical shifts of C-7, C-8 and C-9 were affected by the shielding and desheilding zones of a double bond at C-15. On the basis of the above spectral data, **4** was assigned as 4β,9α,20-trihydroxy-13,15-seco-1,6,15-tigliatriene-3,13-dione.[15,16)]

Compound **5** revealed absorption bands quite similar to those of **4** in the IR spectrum. In the EI-MS spectrum, the molecular ion peak was assigned at *m/z* 306 $[M]^+$, 40 mass unit less than that of **4**, suggesting an isopropyl group is eliminated in **5**. This was confirmed by ^{1}H-NMR (see Experimental) and ^{13}C-NMR (Table 1) spectral data, which elicited signals quite similar to those of **4**, except for the disappearance of a side chain at C-14. In addition, the ^{1}H-NMR spectrum of **5**, with the aid of ^{1}H–^{1}H COSY, showed an upfield shift of H-14 (δ_H 3.39 and 2.18 in **4** and **5**, respectively), along with the appearance of a proton signal at δ_H 2.84, which is coupled with the signal at δ_H 2.18. In the ^{13}C-NMR spectrum, which was analyzed with the aid of HMQC and HMBC experiments, the upfield shift of a C-14 chemical

Table 1. ^{13}C-NMR Spectral Data of Phorbol (**1**) and Its Metabolites (**2**—**6**)

Carbon No.	Phorbol[a,c]	**2**[a,c]	**3**[a,c]	**4**[b,d]	**5**[b,c]	**6**[b,c]
1	161.3	160.0	158.0	158.4	160.0	160.0
2	134.3	136.2	131.4	134.6	135.6	135.3
3	210.8	214.8	211.5	209.8	210.4	210.4
4	74.9	50.3	78.0	73.5	74.5	75.0
5	38.6	34.4	34.4	38.2	38.7	38.0
6	141.9	143.5	140.9	141.3	142.5	140.6
7	131.1	126.7	127.2	126.1	127.4	132.8
8	40.3	42.0	42.0	44.2	40.0	45.0
9	79.8	79.6	79.2	77.2	76.8	78.0
10	58.8	58.1	58.1	58.2	58.5	58.5
11	46.1	45.7	45.7	37.8	43.3	47.0
12	82.0	80.2	80.2	46.3	47.5	79.4
13	63.2	62.8	62.8	208.7	214.2	217.4
14	37.5	36.6	36.6	57.7	43.3	64.8
15	27.0	26.7	26.7	141.1	18.2	72.0
16	24.1	24.3	24.3	116.4	10.2	29.2
17	18.4	17.1	17.1	19.9	68.2	29.7
18	15.4	12.3	12.3	17.8		19.0
19	10.2	10.4	10.4	10.2		10.3
20	68.3	68.5	68.5	67.8		68.3

a) 100 MHz. *b*) 125 MHz. *c*) Measured in CD_3OD. *d*) Measured in $CDCl_3$.

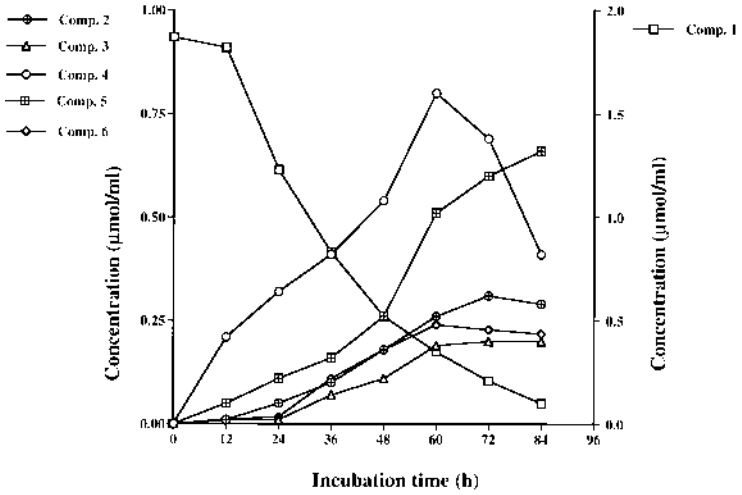

Fig. 1. Time Course of Metabolism of Phorbol (**1**) by Human Intestinal Bacteria

shift (δ_C 43.3) was observed in **5** relative to that of **4** (δ_C 57.7). From these findings, we concluded that the structure of **5** is 4β,9α,20-trihydroxy-15,16,17-trinor-1,6-tigliadiene-3,13-dione.[16)]

The IR spectrum of **6** showed absorption bands similar to that of **1**, together with a strong absorption at 1765 cm^{-1}, indicating a 4-membered ring ketone. In the EI-MS spectrum, the molecular ion peak was assigned at *m*/*z* 346 $[M]^+$ with 18 mass units less than that of **1**, suggesting that **6** is a dehydrating compound of **1**. The ^{1}H- and ^{13}C-NMR spectra indicated a pattern similar to those of **1**. However, the ^{1}H-NMR spectrum of **6** showed a downfield shift of a signal at δ_H 0.68 assigned as H-14 in **1** (H-14 at δ_H 2.45 in **6**). Meanwhile, the ^{13}C-NMR spectrum elicited a highly downfield shift of C-13 due to the formation of a carbonyl group (δ_C 63.2 and 217.4 in **1** and **6**, respectively). Also, the downfield shifts observed at C-8, C-15, C-16 and C-17 could be explained by the desheilding effect of the carbonyl moiety at C-13 (δ_C 40.3, 27.0, 24.1, 18.4 in **1** and 45.0, 72.0, 29.2, 29.7 in **6**). A slight downfield shift of C-18 can be noticed for the same reason mentioned above (δ_C 15.4 and 19.0 in **1** and **6**, respectively). The structure of **6** was further confirmed by the analysis of ^{1}H–^{1}H COSY, DEPT, HMQC and HMBC correlations, and was found to be 4β,9α,20-trihydroxy-14(13→12)-abeo-12α*H*-1,6-tigliadiene-3,13-dione.[15,16)]

Figure 1 shows the time course of the metabolism of **1**, which was completely metabolized to compounds **2**—**6** by a fecal bacterial mixture 84 h after anaerobic incubation. As major metabolites, compounds **4** and **5** were detected first and reached their maxima (43% and 35% of the added substrate, respectively) at 60 and after 84 h, respectively. However, compound **4** seemed to be further metabolized, as shown by its decrease after 60 h. Compound **5** started to increase appreciably after 48 h. Since independent incubation of **4** with an HIB mixture under similar conditions lead to no formation of **5**, it may be concluded that **4** was not a precursor of **5** (data not shown). The other compounds, **2**, **3** and **6**, reached their maxima (17%, 10% and 13% of the added sub-

strate, respectively) 60—72 h after incubation.

No metabolites were obtained by incubation of **1**, either with GAM broth alone or GAM broth with heat-killed bacteria, which indicates the important role of viable bacteria from human feces in the metabolism of **1** under anaerobic conditions. On the other hand, isophorbol (**2**) was not metabolized under the same conditions. The only structural difference between **1** and **2** is a β- or α-oriented hydroxyl group at C-4, respectively.[14] Despite this subtle structural difference between **1** and **2**, they were completely different in their susceptibility to human intestinal bacteria. The difference in metabolic transformation between them may be due to the substrate specificity of the bacterial enzymes responsible for the metabolism of **1**.

The chemical formation of compounds **4**—**6** has been reported by boiling **1** under strong acidic conditions (0.02 N H_2SO_4) (Flaschtentrager reaction),[15,16] while these compounds could not be obtained by incubation of **1** for 3 d in the buffer solution at pH 6 (the same final pH in the incubation mixture in the presence of a fecal intestinal bacteria), indicating that the conversion of **1** was mediated by HIB.

The possible metabolic processes leading to various metabolites **2**—**6** from **1** by HIB is illustrated in Charts 2 and 3. Isophorbol (**2**) may be formed through the isomerization of **1** (Chart 2), while deoxyphorbol (**3**) may be obtained with an enzymatic dehydroxylation process. The formation of compounds **4**—**6** could be explained through concerted mechanisms resulting in the cleavage of a three-membered ring (Chart 3). Chemical cleavage reactions of cyclopropanols in both acidic and basic solution have been reported by DePuy *et al.*[17]

For the purpose of identifying the bacterial strains capable of transforming **1** to its metabolites **2**—**6**, twenty-five defined bacterial strains isolated from human feces were screened for their ability to metabolize **1**. Under anaerobic conditions in GAM broth, only nine of these human intestinal bacteria strains metabolized **1** (Table 2). Six of them had the ability to transform **1** to **4**—**6**, which were *Bifidobacterium adolescentis*, *Clostridium butyricum*, *C. innocuum* ES 24-06, *Peptostreptococcus anaeobius* 0240, *P. intermedius* EBF 77/25, *Proteus mirabillis* S2. While *Fusobacterium nucleatum* G-470, *Ruminococcus* sp. P01-3 had the ability to metabolize **1**

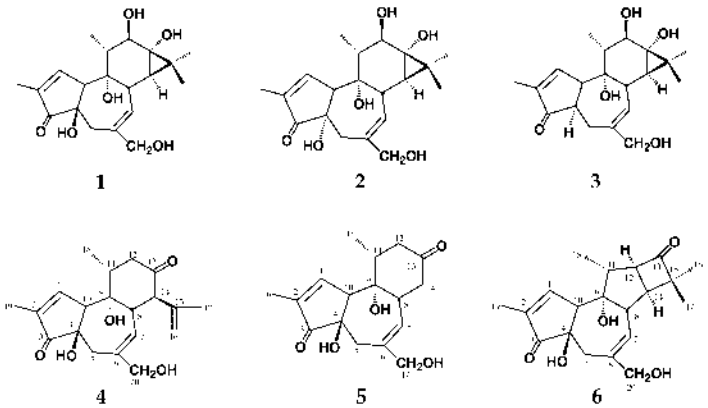

Chart 1. Structures of Phorbol (**1**) and Related Compounds

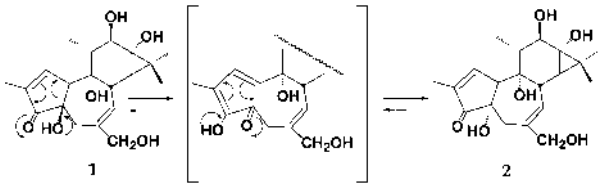

Chart 2. Possible Metabolic Pathways for the Metabolism of Phorbol (**1**) to Isophorbol (**2**) by Human Intestinal Bacteria

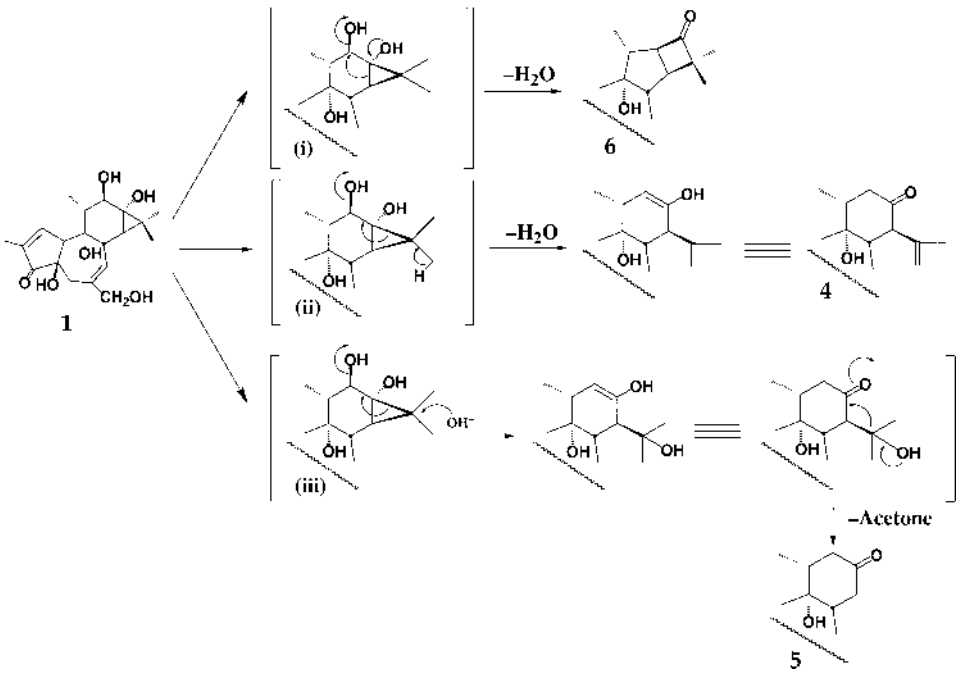

Chart 3. Possible Metabolic Pathways for the Metabolism of Phorbol (**1**) to Compounds **4**—**6** by Human Intestinal Bacteria

Table 2. Phorbol-Metabolizing Activity of Human Intestinal Bacterial Strains

Intestinal bacterial strain	**2**	**3**	**4**	**5**	**6**	Phorbol recovered
Bacteroids fragilis ss *thetaotus*	8.8	0	0	0	0	91.2
Bifidobacterium adolescentis	0	0	51.6	32.8	5.6	10.0
B. breve S-2KZ 1287	0	0	0	0	0	100
B. bifidum aE319	0	0	0	0	0	100
B. longum IV-55	0	0	0	0	0	100
B. pseudolongum PNC-2-9-G	0	0	0	0	0	100
Clostridium butyricum	0	0	46.3	37.2	7.3	9.2
C. innocuum ES 24-06	0	0	26.2	55.3	7.9	10.6
C. innocuum KZ-633	0	0	0	0	0	100
C. perfringens To-23	0	0	0	0	0	100
Escherichia coli 0-12	0	0	0	0	0	100
Fusobacterium nucleatum G-470	0.3	3.3	0	0	0	95.4
Gaffkya anaerobia G-0608	0	0	0	0	0	100
Klebsiella pneumoniae ATCC 13883	0	0	0	0	0	93.6
Lactobacillus acidofiphilus ATCC 4356	0	0	0	0	0	100
L. brevis II-46	0	0	0	0	0	100
L. fermentum ATCC 9338	0	0	0	0	0	100
L. plantarum ATCC 14917	0	0	0	0	0	100
L. xylosus ATCC 155775	0	0	0	0	0	100
Peptostreptococcus anaerobius 0240	0	0	50.4	29.9	8.6	11.1
P. intermedius EBF 77/25	0	0	36.8	49.3	9.8	4.1
Proteus mirabillis S2	0	0	18.6	61.4	6.8	9.2
Ruminococcus sp. P01-3	6.2	1.2	0	0	0	92.6
Streptococcus faecalis II-136	0	0	0	0	0	100
Veillonella parvula ss *parvula* ATCC 10790	0	0	0	0	0	100

Each bacterial species was cultured in GAM broth containing phorbol (10 mg) for 3 d at 37 °C. The culture was extracted with EtOAc and the products were analyzed by TLC densitometry. Each value represents the relative amount (%) of the compound in the reaction mixture.

to **2** and **3**. On the other hand, *Bacteroids fragilis* ss *thetaotus* had the ability to transform **1** to **2** only.

The discovery of any biologically active natural product leads to predictable ancillary research, broadly designed to probe the structural features critical to drug activity. Research approaches usually include total or semi synthesis efforts, alterations in the structures of prototype compounds, and drug metabolism investigations. All of these approaches afford new compounds for biological evaluation, which contribute to our understanding of structural activity relationships. For phorbol (**1**), many semi-synthetic derivatives have been prepared and tested for biological activity. There exists almost no information about the metabolic fate of it at this time.

In our laboratories, initial metabolic investigations of **1** focused on the *in vitro* biotransformation by HIB. This study has provided the first evidence that intestinal bacteria play an important role in the *in vitro* metabolism of **1**. Since the structural complexity of **1** renders total or semi synthetic methods to be difficult, and the biotransformation of physiologically active but toxic compounds may yield metabolites with dissociated physiological activity and toxicity, this material is deemed an excellent candidate for HIB modifications.

Another goal of this study is to use microbial transformation to determine potentially important pathways of bioactivation, bioinactivation and toxicity which may also occur in mammalian species. Further studies relating to the anti-HIV activity of these metabolites are in progress.

Acknowledgement Part of this study was financially supported by Kowa Life Science Foundation (Nagoya City, Japan) and a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

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