Depigmenting Activity and Low Cytotoxicity of Alkoxy Benzoates or Alkoxy Cinnamte in Cultured Melanocytes

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To obtain effective and safe topical depigmenting agents, we synthesized hydroxybenzoates, alkoxybenzoates, and 3,4,5-trimethoxycinnamate containing a thymol moiety and screened then for high-level inhibitory activity against melanin synthesis in cultured melanocytes. Eight compounds were tested for their depigmenting effect and cytotoxicity using a murine melanocyte cell line. We found that 3,4,5-trialkoxybenzoates and 3,4,5-trimethoxycinnamate, synthesized by conjugating 3,4,5-trialkoxybenzoic acids and 3,4,5-trimethoxycinnmic acid with thymol, showed a potent depigmenting effect and low cytotoxicity. Compound 4h, 5-methyl-2-(methylethyl)phenyl (2*E*)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate, showed the most potent depigmenting effect (IC_{50}=10 μM) with low cytotoxicity (IC_{50}=200 μM).

Key words alkoxybenzoate; alkoxycinnamate; thymol; depigmenting effect; low cytotoxicity

Melanogenesis is the process of production of melanin by melanocytes within the skin and hair follicles and is mediated by several enzymes such as tyrosinase, TRP-1, and TRP-2.[1—3] Since tyrosinase[4] is known to be the enzyme responsible for the oxidation of tyrosine, the first and rate-limiting step in melanogenesis, many efforts have been focused on the regulation of tyrosinase activity using small molecular compounds, for example, hydroquinone,[5,6] resorcinol,[7,8] catechol,[9] gentisic acid,[10,11] and gallic acid.[12] The low molecular weight of depigmenting agents is one requirement for efficient delivery into the skin. Their depigmenting effect is closely related to the antioxidant properties of the phenolic hydroxyl group and the cytotoxicity of their intermediates within melanocytes.[13—17] Many compounds and derivatives have been developed but there is still a need to find potent small molecular compounds for depigmentation without compromising cytotoxicity. To meet this need with another avenue of approach, benzoates and cinnamate containing a thymol moiety were synthesized and their depigmenting effects and cytotoxicity were determined in a murine melanocyte cell line.

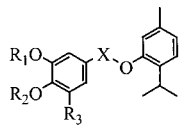
After screening these compounds, alkoxybenzoates and 3,4,5-trimethoxycinnamate showed an unexpectedly strong depigmenting effect with low cytotoxicity, whereas hydroxybenzoates showed a simultaneous depigmenting effect and cytotoxicity, as expected. In this study, a novel way of developing depigmenting agents is presented in which depigmentation is not related to the antioxidant properties of phenolic hydroxyl groups and cytotoxicity within melanocytes.

Results and Discussion

To evaluate synthetic compounds for their potency in melanogenesis inhibition, we compared their activities and cytotoxicity with known depigmenting agents such as hydroquinone, gallic acid, and kojic acid.[18] Structures of compounds **4a**—**h** are shown in Fig. 1 and the synthetic pathways in Fig. 2.

Compounds **4a**—**d**, hydroquinone, gallic acid, and kojic acid were examined for their inhibitory effects against melanin synthesis and cytotoxicity. Hydroquinone showed a potent inhibitory effect (IC_{50}=9 μM) and severe cytotoxicity (IC_{50}=25 μM). Gallic acid showed mild inhibitory activity and cytotoxicity compared with those of hydroquinone. However, kojic acid did not inhibit pigmentation at concentrations up to 2 mM and showed no cytotoxicity at this concentration. Compound **4a**, containing three phenolic hydroxyl groups, exhibited potent inhibitory activity (IC_{50}=10 μM) with cytotoxicity (IC_{50}=33 μM). The inhibitory activity appeared to be related to the cytotoxicity of the phenolic hydroxyl groups. Compound **4b**, containing two phenolic hydroxyl groups, showed similar behavior. However, compound **4c**, containing phenolic hydroxyl group at the *para*-position showed moderate activity (IC_{50}=60 μM) with low cytotoxicity (IC_{50}=300 μM). The reason for this low cytotoxicity might be due to the absence of an oxidation process to yield reactive quinone.[16] Two neighboring methoxyl groups interfere with the transformation of **4c** to its reactive cytotoxic intermediate. Compound **4d**, in which three hydroxyl groups are replaced by methoxyl groups, showed unexpectedly favorable results. The IC_{50} of pigmentation was about 25 μM, but it was not cytotoxic (IC_{50}=120 μM). To expand our understanding of the scope of its activity, we adjusted the thymol moiety and modified the chain length of the alkoxyl group. The results are presented in Table 2.

Thymol showed no depigmenting activity and no cytotoxicity, but trimethoxybenzoic acid showed moderate activity (IC_{50}=80 μM) and cytotoxicity (IC_{50}=80 μM). The activity and cytotoxicity of a simple mixture of thymol and 3,4,5-trimethoxybenzoic acid were similar to those of 3,4,5-trimethoxybenzoic acid. Although the simple mixture was not effective, two components connected with an ester bond



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R_1, R_2, R_3 = H, Me, Et, Pr, Bu. X = CO, CH_2=CH_2CO (*E*)

Fig. 1. Structure of Thymol Esters

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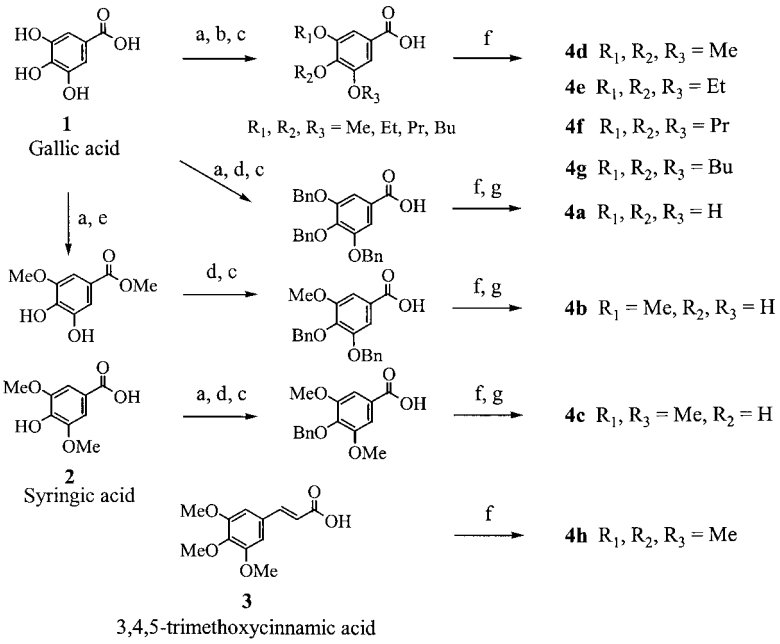

(a) MeOH, TsOH, toluene; (b) alkyl bromide, K_2CO_3, DMF; (c) KOH, H_2O; (d) benzyl bromide, K_2CO_3, DMF; (e) DMS, borax, H_2O; (f) benzenesulfonyl chloride, pyridine, thymol; (g) Pd/C, H_2, EtOAc

Fig. 2. Synthetic Pathway of Thymol Esters (**4a**—**h**)

Table 1. *In Vitro* Assessment of Putative Depigmenting Agents

Compound	Inhibition IC_{50} (μM)	Cytotoxicity IC_{50} (μM)
Hydroquinone	9	25
Gallic acid	35	40
Kojic acid	>2 mM	>2 mM
4a, R_1, R_2, R_3=H; X=CO	10	33
4b, R_1=Me, R_2, R_3=H; X=CO	32	71
4c, R_1, R_3=Me, R_2=H; X=CO	60	300
4d, R_1, R_2, R_3=Me; X=CO	25	120

Table 2. *In Vitro* Assessment of Putative Depigmenting Agents

Compound	Inhibition IC_{50} (μM)	Cytotoxicity IC_{50} (μM)
Thymol	>300	>300
3,4,5-Trimethoxybenzoic acid	>80	>80
Mixture	>80	>80
4d, R_1, R_2, R_3=Me; X=CO	25	120
4e, R_1, R_2, R_3=Et; X=CO	30	>200
4f, R_1, R_2, R_3=Pr; X=CO	12	>200
4g, R_1, R_2, R_3=Bu; X=CO	8	>200
4h, R_1, R_2, R_3=Me; X=CH_2=CH_2CO	10	>200

in general showed strong depigmenting effects with low cytotoxicity. Analysis in terms of cytotoxicity IC_{50}/pigmentation inhibition IC_{50} indicated that the increase in chain length of the alkoxyl group distinctly enhanced depigmentation and safety. Compound **4h**, containing an α,β-unsaturated carbonyl group, showed the most potent depigmenting effect (IC_{50}=10 μM) with low cytotoxicity (IC_{50}=200 μM). The activity was similar to that of hydroquinone. With **4d**—**h**, no effect was detected in the tyrosinase assay and DPPH assay, probably due to the lack of a phenolic hydroxyl group responsible for the antioxidation and radical scavenging activity (Table 3).

Table 3. Mushroom Tyrosinase Inhibition and Radical-Scavenging Effects

Compound	Mushroom tyrosinase inhibition IC_{50} (μM)	DPPH IC_{50} (μM)
Hydroquinone	1.1	38.4
Gallic acid	—	12.8
Kojic acid	21.1	—
4a, R_1, R_2, R_3=H; X=CO	0.8<	22.4
4b, R_1=Me, R_2, R_3=H; X=CO	0.8<	38.3
4c, R_1, R_3=Me, R_2=H; X=CO	—	—
4d, R_1, R_2, R_3=Me; X=CO	—	—
4e, R_1, R_2, R_3=Et; X=CO	—	—
4f, R_1, R_2, R_3=Pr; X=CO	—	—
4g, R_1, R_2, R_3=Bu; X=CO	—	—
4h, R_1, R_2, R_3=Me; X=CH_2=CH_2CO	—	—

—; Not effective.

Conclusions

After screening hydroxybenzoates, alkoxybenzoates, and 3,4,5-trimethoxycinnamate containing a thymol moiety to determine their depigmenting activity using the melan-a assay, we propose that compounds **4e**—**h** are a class of desirable candidates for depigmenting agents. Their structures have no phenolic hydroxyl group. Compounds **4f**—**h** showed potent inhibitory activity similar to that of hydroquinone, with much less cytotoxicity. Hydroquinone has been considered to be the most potent depigmenting agent. However, its depigmenting activity is mediated by oxidation of the phenolic hydroxyl group by tyrosinase, yielding a reactive intermediate responsible for the cytotoxic effects on melanocytes.[16] The depigmenting activity of our new class of molecules may not be related to the direct inhibition of tyrosinase activ-

ity, radical scavenging, and cytotoxicity on melanocytes because of the absence of phenolic hydroxyl groups in their molecular structure. Further studies on their mechanism of action in melanogenesis are underway.

Experimental

Syntheis of Thymol Ester Derivatives In the synthesis of **4a**—**c**, the phenolic hydroxyl groups were protected by a benzyl group and deprotected. All acids were coupled with thymol using the mixed anhydride method[19] as follows. To a solution of 3,4,5-tribenzyloxybenzoic acid (2.0 g, 4.5 mmol) in pyridine (30 ml), benzenesulfonyl chloride (962 mg, 5.4 mmol) was added dropwise at 0 °C. The reaction mixture was allowed to warm up to room temperature and stirred for 30 min. To the stirring reaction mixture, thymol (680 mg, 4.5 mmol) in pyridine (5 ml) was added dropwise for 20 min. After additional stirring for 1 h, the reaction mixture was evaporated *in vacuo* and the residue was extracted with ethyl acetate (300 ml). The ethyl acetate layer was dried over anhydrous magnesium sulfate and evaporated *in vacuo*. The crude product was separated by SiO_2 column chromatography to afford 5-methyl-2-(methylethyl)phenyl 3,4,5-tribenzyloxybenzoate (2.2 g, 85%). Pd/C (10%, 0.5 g) was suspended in a solution of 5-methyl-2-(methylethyl)phenyl 3,4,5-tribenzyloxybenzoate (2.2 g, 3.8 mmol) in ethyl acetate (50 ml), and the mixture was hydrogenated at 2 atm for 3 h. The catalyst was removed by filtration and washed with ethyl acetate (25 ml). The filtrate was dried over anhydrous magnesium sulfate and evaporated *in vacuo*. The crude product was separated by SiO_2 column chromatography to afford **4a** (0.9 g, 78%).

5-Methyl-2-(methylethyl)phenyl 3,4,5-Trihydroxybenzoate, **4a**: IR ν_{max} (KBr) cm^{-1}: 3254, 3100, 2950, 1735. ^{1}H-NMR (DMSO-d_6) δ: 9.48 (bs, 3H), 7.21 (d, 1H, J=7.5 Hz), 7.08 (s, 2H), 7.01 (d, 1H, J=7.5 Hz), 6.89 (s, 1H), 2.99 (m, 1H), 2.26 (s, 3H), 1.12 (d, 6H, J=6.9 Hz). MS (FAB) m/z 303 (M^++1). *Anal.* Calcd for $C_{17}H_{18}O_5$: C, 67.54; H, 6.00. Found: C, 67.50; H, 5.97.

5-Methyl-2-(methylethyl)phenyl 4,5-Dihydroxy-3-methoxybenzoate, **4b**: IR ν_{max} (KBr) cm^{-1}: 3250, 3100, 2949, 1734. ^{1}H-NMR (DMSO-d_6) δ: 9.56 (bs, 2H), 7.29 (s, 1H), 7.25 (d, 1H, J=7.5 Hz), 7.19 (s, 1H), 7.10 (d, 1H, J=7.5 Hz), 6.94 (s, 1H), 3.84 (s, 3H), 2.98 (m, 1H), 2.29 (s, 3H), 1.16 (d, 6H, J=6.9 Hz). MS (FAB) m/z 317 (M^++1). *Anal.* Calcd for $C_{18}H_{20}O_5$: C, 68.34; H, 6.37. Found: C, 68.29; H, 6.31.

5-Methyl-2-(methylethyl)phenyl 4-Hydroxy-3,5-dimethoxybenzoate, **4c**: IR ν_{max} (KBr) cm^{-1}: 3252, 3100, 2951, 1732. ^{1}H-NMR (DMSO-d_6) δ: 9.45 (bs, 1H), 7.36 (s, 2H), 7.25 (d, 1H, J=7.5 Hz), 7.01 (d, 1H, J=7.5 Hz), 6.93 (s, 1H), 3.83 (s, 6H), 2.97 (m, 1H), 2.29 (s, 3H), 1.16 (d, 6H, J=6.9 Hz). MS (FAB) m/z 331 (M^++1). *Anal.* Calcd for $C_{19}H_{22}O_5$: C, 69.07; H, 6.71. Found: C, 68.99; H, 6.70.

5-Methyl-2-(methylethyl)phenyl 3,4,5-Trimethoxybenzoate, **4d**: IR ν_{max} (KBr) cm^{-1}: 3100, 2950, 1730. ^{1}H-NMR ($CDCl_3$) δ: 7.47 (s, 2H), 7.25 (d, 1H, J=7.5 Hz), 7.07 (d, 1H, J=7.5 Hz), 6.93 (s, 1H), 3.95 (s, 3H), 3.94 (2, 6H), 3.01 (m, 1H), 2.34 (s, 3H), 1.23 (d, 6H, J=6.9 Hz). MS (FAB) m/z 345 (M^++1). *Anal.* Calcd for $C_{20}H_{24}O_5$: C, 69.75; H, 7.02. Found: C, 69.71; H, 6.98.

5-Methyl-2-(methylethyl)phenyl 3,4,5-Triethoxybenzoate, **4e**: IR ν_{max} (neat) cm^{-1}: 3101, 2953, 1735. ^{1}H-NMR ($CDCl_3$) δ: 7.36 (s, 2H), 7.24 (d, 1H, J=7.5 Hz), 7.04 (d, 1H, J=7.5 Hz), 6.92 (s, 1H), 4.12 (m, 6H), 2.92 (m, 1H), 2.29 (s, 3H), 1.35 (t, 6H, J=5.4 Hz), 1.27 (t, 3H, J=5.4 Hz), 1.10 (d, 6H, J=6.9 Hz). MS (FAB) m/z 387 (M^++1). *Anal.* Calcd for $C_{23}H_{30}O_5$: C, 71.48; H, 7.82. Found: C, 71.41; H, 7.77.

5-Methyl-2-(methylethyl)phenyl 3,4,5-Tripropoxybenzoate, **4f**: IR ν_{max} (neat) cm^{-1}: 3100, 2950, 1735. ^{1}H-NMR ($CDCl_3$) δ: 7.43 (s, 2H), 7.20 (d, 1H, J=7.5 Hz), 7.02 (d, 1H, J=7.5 Hz), 6.87 (s, 1H), 4.02 (m, 6H), 3.01 (m, 1H), 2.34 (s, 3H), 1.90 (m, 6H), 1.22 (d, 6H, J=6.9 Hz), 1.06 (m, 9H). MS (FAB) m/z 429 (M^++1). *Anal.* Calcd for $C_{26}H_{36}O_5$: C, 72.87; H, 8.47. Found: C, 72.81; H, 8.42.

5-Methyl-2-(methylethyl)phenyl 3,4,5-Tributoxybenzoate, **4g**: IR ν_{max} (neat) cm^{-1}: 3100, 2952, 1734. ^{1}H-NMR ($CDCl_3$) δ: 7.43 (s, 2H), 7.20 (d, 1H, J=7.5 Hz), 7.02 (d, 1H, J=7.5 Hz), 6.90 (s, 1H), 4.05 (m, 6H), 3.01 (m, 1H), 2.34 (s, 3H), 1.80 (m, 6H), 1.48 (m, 6H), 1.23 (d, 6H, J=6.9 Hz), 0.97 (m, 9H). MS (FAB) m/z 471 (M^++1). *Anal.* Calcd for $C_{26}H_{36}O_5$: C, 74.01; H, 8.99. Found: C, 73.97; H, 8.93.

5-Methyl-2-(methylethyl)phenyl (2*E*)-3-(3,4,5-Trimethoxyphenyl)prop-2-enoate, **4h**: IR ν_{max} (KBr) cm^{-1}: 3100, 2950, 1707, 1620. ^{1}H-NMR ($CDCl_3$) δ: 7.80 (1H, d, J=15.9 Hz), 7.25 (s, 1H), 7.20 (1H, d, J=7.5 Hz), 7.01 (1H, J=7.5 Hz), 6.90 (s, 1H), 6.82 (s, 2H), 6.60 (1H, d, J=15.9 Hz), 3.91 (s, 3H), 2.99 (s, 6H), 3.01 (m, 1H), 2.33 (s, 3H), 1.22 (d, 6H, J=6.9 Hz). MS (FAB) m/z 371 (M^++1). *Anal.* Calcd for $C_{22}H_{26}O_5$: C, 71.33; H, 7.07. Found: C, 71.29; H, 7.01.

Cell Culture Melan-a melanocytes are a highly pigmented, immortalized normal murine melanocyte cell line derived from C57BL/6 mice. The melan-a melanocytes used in this study were obtained from Dr. Dorothy Bennett (St. George's Hospital, London, U.K.). Cells were grown and maintained at 37 °C in an atmosphere of 95% air, 5% CO_2 in RPMI-1640 (Bio Whittaker, Walkersville, MA, U.S.A.) supplemented to a final concentration of 10% heat-inactivated fetal bovine serum, penicillin 5 units/ml, streptomycin 5 μg/ml and 200 nM phorbol 12-myristate 13-acetate. Cells were passaged every 3 d with a maximal passage number of 33. Confluent monolayers of melanocytes were harvested with a mixture of 0.05% trypsin and 0.53 mM EDTA (Gibco BRL, Grand Island, NY, U.S.A.).

Measurements of Melanin Content and Cell Viability Melanin content and cell number were measured in melan-a melanocytes. One hundred thousand cells were seeded into each well of 24-well plates and compounds were added to triplicate cultures. Medium was changed daily, and after 4 d of culture, the cells were lysed with 1 N NaOH 1 ml and pipetted repeatedly to homogenize. For analysis, 200 μl of each crude cell extract was transferred into 96-well plates. The relative melanin content was measured at 400 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tex Instruments). Cell viability was determined using the crystal violet assay. The culture medium was removed from the 24-well culture plates and replaced with 0.5 ml of 0.1% crystal violet in 10% ethanol per well. The plates were stained for 5 min at room temperature and rinsed four times. The crystal violet retained by adherent cells was extracted with 1 ml of 95% ethanol. Absorbance was determined at 540 nm using an ELISA reader.

Mushroom Tyrosinase Assay Mushroom tyrosinase, L-tyrosine, and L-DOPA were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Tyrosinase activity was determined using the a method of Pomerantz[20] with minor modification. Twenty-five microliters of 0.5 mM L-DOPA, 25 μl of 10 mM L-tyrosine, 875 μl of 50 mM phosphate buffer (pH 6.5), and 25 ml of test sample solution were mixed. Then 50 μl of mushroom tyrosinase (1600 U/ml) was added. The amount of dopachrome produced in the reaction mixture was determined against a blank (solution without enzyme) at 475 nm (OD_{475}) using a spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

DPPH Assay DPPH reagent was prepared at a DPPH concentration of 80 μg/ml in MeOH. A test sample (50 μl) was dissolved in DMSO and mixed with 100 mM Tris–HCl buffer (pH 7.4, 50 μl), distilled water, and 400 ml of DPPH ethanolic solution (50 μl). The mixture was shaken well and allowed to stand for 20 min in the dark. The absorbance was measured at 515 nm using an Elx800 microtiter plate reader (Bio-Tek Instruments, Vermont, U.S.A.).

References and Notes

1) Sturm R. A., *Mutat. Res.*, **422**, 69—76 (1998).
2) Marmol V. D., Ito S., Jackson I., Vachtenheim J., Berr P., Ghanem G., Morandini R., Wakamatsu K., Huez G., *FEBS Lett.*, **327**, 307—310 (1993).
3) Amae S., Yasumoto K., Takeda K., Udono T., Takahashi K., Shibahara S., *Biochim. Biophys. Acta*, **1492**, 505—508 (2000).
4) Marmol V. D., Beermann F., *FEBS Lett.*, **381**, 165—168 (1996).
5) Jimbow K., Obata H., Pathak M. A., Fitzpatrick T. B., *J. Invest. Dermatol.*, **62**, 436—449 (1974).
6) Patick E., Juberg D. R., O'Donoghue J., Maibach H. I., *Food Chem. Toxicol.*, **37**, 169—175 (1999).
7) Coupvray A. L., Sevran H., U.S. Patent 5468472 (1995).
8) Katagiri T., Okubo T., Oyobikawa M., Futaki K., Sagaku M., "Proceedings of the 20th IFSCC Congress," Cannes, volume 1, 1998, pp. 93—102.
9) Ito S., Kato T., Ishikawa K., Kasuga T., Jimbow K., *Biochem. Pharmacol.*, **36**, 2007—2011 (1987).
10) Dooley T. P., Gadwood R. C., Kilgore K., Thomasco L. M., *Skin Pharmacol.*, **7**, 188—200 (1994).
11) Curto E. V., Kwong C., Hermersdorfer H., Glatt H., Santis C., Virador V., Hearing V. J., Dooley T. P., *Biochem. Pharmacol.*, **57**, 663—672 (1999).
12) Hase T., Murase T., Shibuya J., Tokimitsu I., Nishizawa Y., Jpn. Patent 08283137 (1996).
13) Riley P. A., Sawyer B., Wolff M. A., *J. Invest. Dermatol.*, **64**, 86—89 (1975).
14) Alena F., Jimbow K., Ito S., *Cancer Res.*, **50**, 3743—3747 (1990).
15) Passi S., Picardo M., Nazzaro-Porro N., *Biochem. J.*, **245**, 537—542

(1987).
16) Thorneby-Addersson K., Sterner O., Hansson C., *Pigment Cell Res.*, **13**, 33—38 (2000).
17) Tayama K., Takahama M., *Pigment Cell Res.*, **15**, 447—453 (2002).
18) Ohyama Y., Mishima Y., *Fragrance J.*, **6**, 53—58 (1990).
19) Jaouadi M., Martinex J., Castro B., *J. Org. Chem.*, **52**, 2364—2367 (1987).
20) Pomerantz S. H., *J. Biol. Chem.*, **238**, 2351—2357 (1963).