After filtration, the absorbance of the saturated solution was determined at 490 nm.

Potentiometric Titrations were carried out automatically in thermostated (25.00 ± 0.05 °C) titration cells, under an atmosphere of nitrogen.

Registry No. 1, 26921-58-4; **2**, 75265-14-4; (at)(PMAA) (homopolymer), 25087-26-7; (st)(PMAA) (homopolymer), 25750-36-1; poly(acrylic acid) (homopolymer), 9003-01-4; poly(*N*-vinylpyrrolidone) (homopolymer), 9003-39-8; Orange OT, 2646-17-5.

Absolute Configuration of Epoxyeicosatrienoic Acids (EETs) Formed during Catalytic Oxygenation of Arachidonic Acid by Purified Rat Liver Microsomal Cytochrome P-450[1]

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Abstract: Incubation of arachidonic acid with a reconstituted enzymatic system containing a purified preparation of the major, phenobarbital-inducible form of rat liver microsomal cytochrome P-450, NADPH, cytochrome b_5, and NADPH-cytochrome P-450 reductase affords as the principal products four regioisomeric *cis*-epoxides: 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids (EETs). Their absolute configurations were established by conversion to the corresponding hydroxyeicosatetraenoic acid (HETE) methyl esters, derivatization with dehydroabiethylisocyanate, and chromatographic analysis. Except for 5,6-EET, the cytochrome P-450 catalyzed epoxidation is highly enantioselective.

A thorough knowledge of the arachidonic acid cascade is vital to our understanding of polyunsaturated fatty acid metabolism and its relationship to physiological and disease processes.[3] Recently, evidence has been presented from studies using reconstituted[4] and microsomal cytochrome P-450[5] as well as intact cells[6] for an additional route to eicosanoids. Designated the epoxygenase pathway,[7a] this route is catalyzed by cytochrome P-450 and is distinct from the well-established lipoxygenase and cyclooxygenase pathways of the arachidonate cascade. In addition to various lipoxygenase-type[8] and ω/ω - 1 oxidation products,[9] the epoxygenase pathway generates several novel metabolites,[4-6] some of which have been shown by us to have potent biological activity in vitro.[7] The validity of these observations has been confirmed with the in vivo detection of epoxygenase metabolites in mammalian tissue.[10]

As part of a comprehensive study of polyunsaturated fatty acid metabolism by cytochrome P-450 and as an aid to structural studies of other epoxygenase metabolites, we report herein the absolute configuration of the arachidonate metabolites 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid (EETs) produced by

Scheme I

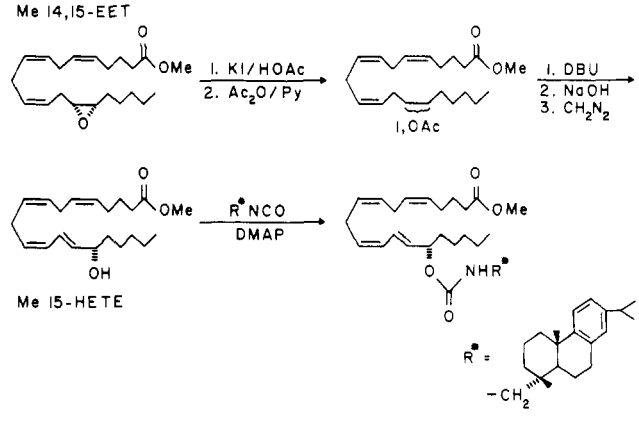

Table I. Enantioselectivity of Arachidonate Epoxygenation

EET	*R*, *S* (%)	*S*, *R* (%)
5, 6	61	39
8, 9	97	3
11, 12	3	97
14, 15	80	20

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(2) (a) Molecular Genetics. (b) Internal Medicine. (c) Biochemistry.

(3) Recent reviews: Nelson, N. A.; Kelly, R. C.; Johnson, R. A. *Chem. Eng. News* **1982**, *Aug. 16*, 30-44. Samuelsson, B. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 902-910. Bailey, D. M.; Casey, F. B. *Ann. Rep. Med. Chem.* **1982**, *17*, 203-217.

(4) (a) Capdevila, J.; Parkhill, L.; Chacos, N.; Okita, R.; Masters, B. S. S.; Estabrook, R. W. *Biochem. Biophys. Res. Commun.* **1981**, *101*, 1357-1363. (b) Oliw, E. H.; Guengerich, F. P.; Oates, J. A. *J. Biol. Chem.* **1982**, *257*, 3771-3781.

(5) (a) Capdevila, J.; Chacos, N.; Werringloer, J.; Prough, R. A.; Estabrook, R. W. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 5362-5366. (b) Chacos, N.; Falck, J. R.; Wixtrom, C.; Capdevila, J. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 916-922. (c) Morrison, A. R.; Pascoe, N. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 7375-7378.

(6) Oliw. E. H.; Moldéus, P. *Biochim. Biophys. Acta* **1982**, *721*, 135-143.

(7) (a) Capdevila, J.; Chacos, N.; Falck, J. R.; Manna, S.; Negro-Vilar; A.; Ojeda, S. R. *Endocrinology* **1983**, *113*, 421-423. (b) Snyder, G. D.; Capdevila, J.; Chacos, N.; Manna, S.; Falck, J. R. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 3504-3507. (c) Falck, J. R.; Manna, S.; Moltz, J.; Chacos, N.; Capdevila, J. *Biochem. Biophys. Res. Commun.* **1983**, *114*, 743-749.

(8) Capdevila, J.; Marnett, L. J.; Chacos, N.; Prough, R. A.; Estabrook, R. W. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 767-770.

(9) Manna, S.; Falck, J. R.; Chacos, N.; Capdevila, J. *Tetrahedron Lett.* **1983**, *24*, 33-36 and references cited therein.

(10) Capdevila, J.; Estabrook, R. W.; Napoli, J. L.; Pramanik, B.; Manna, S.; Falck, J. R. *Arch. Biochem. Biophys.*, in press.

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a reconstituted enzymatic system containing a purified preparation of the major, phenobarbital-inducible form of rat liver microsomal cytochrome P-450.

Results and Discussion

[1-^{14}C]Arachidonic acid was incubated[4a] with purified[11] rat liver microsomal cytochrome P-450 (18 nmol/mg of protein) in a reconstituted system of purified NADPH-cytochrome P-450 reductase, cytochrome b_5, and NADPH in the presence of dilauroylglycero-3-phosphocholine for 10 min at 30 °C. High-pressure liquid chromatography[5b] (HPLC) of the ether-soluble products provided 5,6-, 8,9-, 11,12-, and 14,15-EET as the predominate products in a 1.5:1.0:1.6:5.0 ratio, respectively. Following esterification (CH_2N_2, Et_2O/MeOH, 0 °C), the individual Me-EETs were mixed with 4–5 mg of the corresponding unlabeled synthetic[12] Me-EET as carrier. These were converted[13] to the respective hydroxyeicosatetraenoic acids (HETEs) in 40–80% overall yield by sequential epoxide cleavage in acetic acid saturated aqueous potassium iodide–tetrahydrofuran, acetylation, elimination of the elements of HI from the resultant mixture of regioisomeric iodoacetates using excess 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), saponification, and chromatographic purification (Scheme I, shown for Me-14,15-EET → 15-HETE). Only the homoallylic iodide obtained from 5,6- and 14,15-EET eliminated giving 5- and 15-HETE, respectively; the other regioisomer was unreactive under the reaction conditions and regenerated EET upon saponification. Since both regioisomeric iodoacetates derived from 8,9-EET were reactive, equal amounts of 8- and 9-HETE were obtained and resolved by HPLC. Similarly, 11,12-EET evolved 11- and 12-HETE.[14]

With the adoption of the method of Corey and Hashimoto for the resolution of (*R*)- and (*S*)-HETE enantiomers,[16] the above HETEs were esterified (CH_2N_2) and derivatized with dehydroabietylisocyanate. After chromatographic purification, the diastereomeric urethanes from each Me-HETE were resolved by HPLC (μ-Porasil) with baseline separation, collected individually (UV monitoring, 254 nm), and the relative amounts were determined by scintillation counting (Table I). Stereochemistry was established by HPLC comparison with HETE urethane standards of known absolute configuration. Treatment of synthetic (racemic) methyl 5-, 8-, 9-, 11-, 12-, and 15-HETE as above afforded exactly equal amounts of (*R*) and (*S*) isomers indicating no preferential reaction with the chiral isocyanate reagent. Analysis of 15(*S*)-HETE from incubation of arachidonic acid with soybean lipoxidase[17] and 12-(L)-HETE (New England Nuclear) by the same procedure gave 86% *S* (14% *R*) and >95% *S*, respectively.

Table I shows that the cytochrome P-450 catalyzed epoxidation of the 8,9, 11,12, and 14,15 olefins of arachidonic acid proceeds with a high degree of enantiofacial selectivity. A lesser selectivity is seen at the 5,6 olefin. These results, in conjunction with published data,[4-6,8] clearly indicate the enzymatic nature of the reaction and further suggest that the fatty acid molecule is at least partially held in a preferred orientation during oxidation.

To our knowledge, these data represent the highest enantioselectivity ever observed for the oxidation of an unbiased, acyclic system by cytochrome P-450 and may prove useful in elucidating the active site topography of this enzyme.

Experimental Section

General. All reactions were conducted under an argon or nitrogen atmosphere. Eicosanoids were stored in benzene at −20 °C under argon. ^{1}H NMR spectra were measured at 90 MHz on a JEOL FX-90Q spectrometer in $CDCl_3$ with tetramethylsilane as internal standard. Low- (CI/EI) and high-resolution mass spectra were obtained on a Finnigan 4021 and MAT-711, respectively. Unless otherwise stated, all products were oils. Arachidonic (99%) and homo-γ-linolenic acid were purchased from Nu Chek Prep, Inc. (Elysian, MN); [1-^{14}C]arachidonic acid was from Amersham (Arlington Heights, IL). Soybean lipoxidase (Type I) was from Sigma Chemical Co. (St. Louis, MO). Incubations of arachidonic acid with purified cytochrome P-450 and product isolation were performed exactly as previously described.[5b] Thin-layer chromatography (TLC) was conducted in the dark on precoated 20 × 20 cm E. Merck silica gel 60 plates (0.25 mm thickness) in argon flushed tanks equilibrated with solvent.

Conversion of Me-EETs to Me-HETEs. The preparation[13] of Me-5-HETE from Me-5,6-EET is representative. A mixture of racemic, unlabeled Me-5,6-EET (4 mg) and radiolabeled cytochrome P-450 derived Me-5,6-EET (54.9 Ci/mol, 47 × 10^3 dpm) in tetrahydrofuran (THF) (0.5 mL) was added to a 0 °C solution of acetic acid (1 mL) and saturated aqueous potassium iodide (0.2 mL) and stirred at 4 °C protected from light. After 16 h, the organic solvents were evaporated and the residue was extracted with Et_2O (3 × 2 mL). The combined organic extracts were washed with H_2O, 5% $Na_2S_2O_3$ solution, 5% $NaHCO_3$ solution, and brine, dried, and evaporated. The resultant regioisomeric iodohydrins could be separated by careful chromatography (TLC, SiO_2, Et_2O/hexane 1:1, R_f 0.27 and 0.29) in a 1.5:1 ratio but were routinely used as a mixture in the next step.

To the above mixture in dry CH_2Cl_2 (0.5 mL) at 0 °C was added pyridine (0.2 mL) followed by addition of Ac_2O (0.1 mL). After the mixture was stirred at room temperature in the dark overnight, ice chips were added and the products extracted with Et_2O (3 × 2 mL). The combined ethereal extracts were washed with H_2O, 5% $NaHCO_3$ solution, and brine, dried, and evaporated.

Without purification, the preceding mixture of iodoacetates in dry benzene (0.8 mL) was treated with 4–6 equiv of 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) for 20 h. Powdered $CuSO_4$ was added, and after the reaction mixture was stirred for 30 min it was quickly passed through a bed of silica gel using Et_2O/hexane (3:1). Chromatographic separation (TLC, SiO_2, 1% MeOH/CH_2Cl_2) afforded the unreacted 5-iodo-6-acetate isomer (R_f ~0.57), which can be recycled to 5,6-EET by saponification, and Me-5-HETE acetate (R_f ~0.54), spectrally and chromatographically identical with an authentic sample. NMR δ 0.92 (3 H, t), 1.16–1.80 (10 H, m), 1.88–2.48 (7 H, m, including acetate methyl singlet), 2.68–3.08 (4 H, m), 3.68 (3 H, s), 5.16–5.78 (7 H, m, olefins and C-5 methine), 5.94 (1 H, dd, J ~ 10, 10 Hz, C-8), 6.54 (1 H, dd, J ~ 10, 15 Hz, C-7).

Saponification in THF (2 mL) and H_2O (1 mL) with 4 equiv of NaOH for 12 h followed by extractive isolation at pH 3 and treatment with diazomethane furnished, after purification (TLC, SiO_2, Et_2O/hexane 2:1, R_f ~0.43), Me-5-HETE (40% overall yield from Me-5,6-EET) indistinguishable (except for optical rotation) from a known sample.[16]

Me-8,9-EET to Me-8- and Me-9-HETE. Me-8- and Me-9-HETE were prepared as above from a mixture of carrier and radiolabeled Me-8,9-EET in 40% overall yield for each isomer. Iodohydrins: TLC, SiO_2, Et_2O/hexane 1:1, R_f ~0.46. Iodoacetates: TLC, SiO_2, Et_2O/hexane 1:2, R_f ~0.36. Mixture of Me-8- and Me-9-HETE acetates: TLC, SiO_2, 1% MeOH/CH_2Cl_2, R_f ~0.55. Mixture of 8- and 9-HETE: TLC, SiO_2, 5% MeOH/CH_2Cl_2, R_f ~0.15. The HETEs were separated by HPLC on a Waters μ-Porasil analytical column using a gradient solvent system of 0.5% *i*-PrOH/0.1% AcOH/99.4% hexane to 1.5% *i*-PrOH/0.1% AcOH/98.4% hexane over 30 min, with a flow rate of 3 mL/min. Retention time: 19.2–20.8 and 21.8–23.2 min for 9- and 8-HETE, respectively. Me-8- and Me-9-HETE: TLC, SiO_2, Et_2O/hexane 1:1, R_f ~0.32.

Me-11,12-EET to Me-11- and Me-12-HETE. Me-11- and Me-12-HETE were prepared as above from a mixture of carrier and radiolabeled Me-11,12-EET in 42% and 38% overall yield, respectively. Iodohydrins: TLC, SiO_2, Et_2O/hexane 1:1, R_f ~0.43. Iodoacetates: TLC, SiO_2, Et_2O/hexane 1:2, R_f ~0.45. Mixture of 11- and 12-HETE acetates: TLC, SiO_2, 1% MeOH/CH_2Cl_2, R_f ~0.57. Mixture of 11- and 12-HETE: TLC, SiO_2, 5% MeOH/CH_2Cl_2, R_f ~0.17. HETEs separated on a Waters μ-Porasil analytical column using the gradient system described above. Retention time: 7.7–8.5 and 13.3–14.2 min for 12- and

(11) Imai, Y.; Sato, R. *Biochem. Biophys. Res. Commun.* **1974**, *66*, 8–14.
(12) (a) Corey, E. J.; Niwa, H.; Falck, J. R. *J. Am. Chem. Soc.* **1979**, *101*, 1586–1587. (b) Corey, E. J.; Marfat, A.; Falck, J. R.; Albright, J. O. *Ibid.* **1980**, *102*, 1433–1435. (c) Falck, J. R.; Manna, S. *Tetrahedron Lett.* **1982**, *23*, 1755–1756.
(13) Modified from the procedure of Corey et. al.: Corey, E. J.; Albright, J. O.; Barton, A. E.; Hashimoto, S.-I. *J. Am. Chem. Soc.* **1980**, *102*, 1435–1436.
(14) As a control, enantiomerically homogeneous methyl *cis*-9,10-oxido-*trans*-12-octadecenoate (prepared from methyl ricinoleate by *t*-BuOOH/VO(acac)$_2$ epoxidation, tosylation, and elimination with DBU in benzene at reflux) was converted by this general procedure to (+)-methyl dimorphecolate whose specific rotation and spectroscopy were identical with literature values.[15] Chromatographic analysis of the dehydroabietylurethane derivative showed a single isomer under conditions which resolve the (*R*)- and (*S*)-urethanes.
(15) Applewhite, T. H.; Binder, R. G.; Gaffield, W. *J. Org. Chem.* **1967**, *32*, 1173–1178.
(16) Corey, E. J.; Hashimoto, S.-I. *Tetrahedron Lett.* **1981**, *22*, 299–302.
(17) Baldwin, J. E.; Davies, D. I.; Hughes, L.; Gutteridge, N. J. A. *J. Chem. Soc., Perkin Trans. 1* **1979**, 115–121.

Table II

urethane	TLC R_f (SiO_2,Et_2O/hexane 1:2)	HPLC retention time, min
5	0.27 and 0.30	24.6-26.2
8	0.36	25.8-26.8
9	0.34	26.2-27.4
11	0.38	26.0-26.8
12	0.38	25.6-26.6
15	0.40	24.6-26.2

Table III

urethane	retention time, min		solvent	flow rate, mL/min
	R	*S*		
5	5.9	7.2	0.4% *i*-PrOH/0.1%HOAc/hexane	3.0
8	23.6	20.2	0.25% *i*-PrOH/hexane	1.0
9	42.2	46.2	0.25% *i*-PrOH/hexane	0.6
11	16.9	19.3	0.2% *i*-PrOH/hexane	2.0
12	186.6	172.4	0.18% *i*-PrOH/hexane	0.6
15	16.9	15.2	0.2% *i*-PrOH/hexane	1.5

11-HETE, respectively. Me-11- and Me-12-HETE: TLC, SiO_2, Et_2O/hexane 1:1, $R_f \sim 0.35$.

Me-14,15-EET to Me-15-HETE. Me-15-HETE was prepared as above from a mixture of carrier and radiolabeled Me-14,15-EET in 40% yield overall. Iodohydrins: TLC, SiO_2, Et_2O/hexane 1:1, $R_f \sim 0.42$ and 0.45, 1.5:1 ratio, respectively. Iodoacetates: TLC, SiO_2, Et_2O/hexane 1:2, $R_f \sim 0.38$. Me-15-HETE acetate and unreacted iodoacetate regioisomer were separated chromatographically: TLC, SiO_2, 1% MeOH/CH_2Cl_2, $R_f \sim 0.67$ and 0.69, respectively. NMR analysis revealed that the Me-15-HETE acetate was contaminated with an isomeric diene which was removed chromatographically after saponification and re-esterification (CH_2N_2). Me-15-HETE and isomeric diene: TLC, SiO_2, Et_2O/hexane 1:1, $R_f \sim 0.31$ and 0.27, respectively.

Me-HETE Urethanes: General Procedure. The Me-HETEs were derivatized with dehydroabietylisocyanate as described by Corey and Hashimoto[16] in 70-90% yield after TLC purification (SiO_2, Et_2O/hexane 1:2). The TLC products were further purified on a Waters μ-Bondapak C_{18} analytical column using a solvent program of isocratic 80% CH_3CN/19.9% H_2O/0.1% AcOH for 10 min and then a gradient over 20 min to 99.9% CH_3CN/0.1% HOAc at a flow rate of 2 mL/min.

The spectral data for Me-15-HETE urethane are representative of the urethane derivatives. NMR δ 0.84-2.20 (37 H, complex m), 2.36 (2 H, t, $J \sim 7$ Hz), 2.68-3.20 (8 H, m), 3.68 (3 H, s), 4.60-4.88 (1 H, m, C-15 methine), 5.16-5.80 (6 H, m), 5.96 (1 H, dd, $J \sim 10$, 10 Hz, C-12), 6.52 (1 H, dd, $J \sim 10$, 15 Hz, C-13), 6.82-7.22 (3 H, m, ArH); mass spec PCI (CH_4) *m/e* 646 (M + 1, 5%), 317 (75%), 286 (100%); high-resolution mass spec calcd for $C_{42}H_{32}NO_4$ 645.4757, found 645.4763.

Stereochemical Analysis. The purified diastereomeric urethane derivatives were resolved with baseline separation on a Waters μ-Porasil analytical column with UV monitoring (254 nm) with use of an isocratic solvent system.

Comparisons with Me-HETE urethane standards of known configuration were used to determine the absolute configuration of one side of the EET epoxide. Since the EETs are *cis*-epoxides, the configuration of the opposite side of the epoxide could be deduced.

Standards. 5-HETE derivatives were prepared and resolved by the method of Corey and Hashimoto.[16] Authentic 12(L)-HETE was purchased from New England Nuclear and 15(*S*)-HETE was obtained from an incubation of arachidonic acid with soybean lipoxidase.[17] Both were derivatized as described above. For analysis of the Me-8-HETE urethane, the sample was reduced (1 atm) over Pd/C in EtOH and compared wth the saturated urethane of methyl 8(*S*)-hydroxyeicosatrienoate prepared by incubation of homo-γ-linolenic acid with New Zealand White rabbit leukocytes.[18]

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(18) Borgeat, P.; Hamberg, M.; Samuelsson, B. *J. Biol. Chem.* **1976**, *251*, 7816-7820. See correction: *Ibid.* **1977**, *252*, 8772.

Phosphorus-31 NMR and Kinetic Studies of the Formation of Ortho-, Pyro-, and Triphosphato Complexes of *cis*-Dichlorodiammineplatinum(II)

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Abstract: The reactions of *cis*-dichlorodiammineplatinum(II) with pyro- and triphosphate follow biphasic kinetics over the temperature range 25-40 °C in 0.5 M $NaClO_4$ (pH 6.0-8.4). The initial step is a composite of aquation and monodentate phosphate complexation. The final step of the reaction is attributed to chelation by the phosphate ligands. The rate constants for aquation, monodenatate complexation, and chelation at 25 °C and pH 6.0 are 2.8×10^{-5} s^{-1}, 7.3×10^{-4} M^{-1} s^{-1}, and 2.9×10^{-5} s^{-1} for triphosphate and 2.9×10^{-5} s^{-1}, 1.4×10^{-3} M^{-1} s^{-1}, and 4.5×10^{-5} s^{-1} for pyrophosphate, respectively. The activation parameters (ΔH^*, kcal mol^{-1}, ΔS^*, cal deg^{-1} mol^{-1}) associated with the aquation, monodentate complexation, and chelation are 19.8, -14; 15.6, -20; and 11.8, -40 for triphosphate ion and 19.7, -14; 15.3, -21; and 11.5, -37 for pyrophosphate ion, respectively. Both monodentate and chelate complexes of pyro- and triphosphate were observed by phosphorus-31 NMR spectroscopy. The chemical shifts of coordinated phosphate groups of chelates are about 4-8 ppm downfield relative to the free phosphates while the monodentate phosphato complexes have smaller (2-3.5 ppm) coordination chemical shifts. The reaction of orthophosphate ion with *cis*-dichlorodiammineplatinum(II) at pH 6.0 in 0.5 M $NaClO_4$ yielded a blue species which has been characterized by formation kinetics, analytical data, redox titration, and ESR spectroscopy.

Many metal ions are known to form stable complexes with a variety of phosphate ligands.[2] Cobalt(III) complexes with phosphate ligands have been investigated in detail in this laboratory[3-5] and elsewhere.[6] Various binding modes of orthophosphate and polyphosphates have been reported. For example, triphosphate coordinates to cobalt(III) in both monodentate and bidentate fashions.[3-5] Two monodentate isomers containing triphosphate coordinated through the terminal phosphate group (the γ-isomer) and through the middle phosphate group (the β-isomer) have been isolated and characterized by X-ray crys-

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