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PAPER

Chemoenzymatic synthesis of (2*R*,3*R*,4*R*)-dehydroxymethylepoxyquinomicin (DHMEQ), a new activator of antioxidant transcription factor Nrf2

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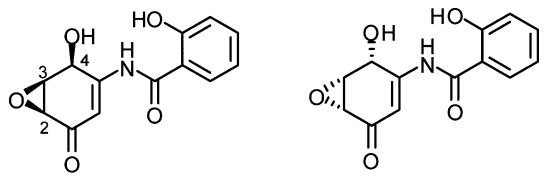
Dehydroxymethylepoxyquinomicin (DHMEQ, **1a**) is a specific and potent inhibitor of NF-κB, and it is now being developed as an anti-inflammatory and anticancer agent. While previously only the (2*S*,3*S*,4*S*)-form had been available from the racemate by using lipase-catalyzed enantioselective resolution, in the present study a new route for production of the (2*R*,3*R*,4*R*)-form was established by use of a chemoenzymatic approach. (1*R**,2*R**,3*R**)-2,3-Epoxy-5-*N*-[(2-hydroxybenzoyl)amino]-4,4-dimethoxycyclohex-5-en-1-ol (**2a**) was hexanoylated on both secondary and phenolic hydroxy groups, and subjected to *Burkholderia cepacia* lipase-catalyzed hydrolysis. The reaction proceeded in a highly enantioselective manner ($E > 500$) to give (1*S*,2*S*,3*S*)-**2a** in an enantiomerically pure state. Several chemical steps of transformation from the enzyme reaction product gave (2*R*,3*R*,4*R*)-DHMEQ (**1a**) without any loss of stereochemical purity. Moreover, we newly found that (2*R*,3*R*,4*R*)-DHMEQ activated Nrf2, which is a transcription factor that induces the expression of multiple antioxidant enzymes. It activated Nrf2 in a promoter reporter assay. It also increased the expression of target antioxidant proteins and cancelled ROS-induced cell death in a neuronal cell line. Thus, (2*R*,3*R*,4*R*)-DHMEQ was efficiently prepared by a newly designed route using lipase, and it may be useful as a new anti-inflammatory agent.

Introduction

NF-κB is a transcription factor that promotes expression of many inflammatory cytokines and anti-apoptosis proteins. Therefore, excess activation of NF-κB in natural immune cells often causes inflammation, and in cancer cells increases malignancy. Earlier we designed and synthesized a new NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ, **1a**, Fig. 1), based on the structure of the antibiotic epoxyquinomicin C.[1] DHMEQ shows potent anti-inflammatory and anticancer activities in cultured cells and in animal models.[2] Racemic DHMEQ can be synthesized from 2,5-dimethoxyaniline in 5 steps, and after enantiomeric separation the (2*S*,3*S*,4*S*)-(−)-form shows 10 times stronger activity towards inhibiting NF-κB than its enantiomer.[3] (2*S*,3*S*,4*S*)-DHMEQ was shown to bind directly to a specific cysteine residue in NF-κB components with a stoichiometry of 1 : 1.[4] For the preparation of (2*S*,3*S*,4*S*)-DHMEQ we succeeded in an effective synthesis employing the hydrolysis of DHMEQ dihexanoyl ester with lipase.[5] Fortunately, we could obtain (2*S*,3*S*,4*S*)-DHMEQ while leaving the monoester of (2*R*,3*R*,4*R*)-DHMEQ.



(2*S*,3*S*,4*S*)-**1a** (2*R*,3*R*,4*R*)-**1a**

Fig. 1 Structures of (2*S*,3*S*,4*S*)- and (2*R*,3*R*,4*R*)-**1a**.

Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) is a transcription factor that induces the expression of many antioxidant enzymes, including heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), and glutamate cysteine ligase catalytic (GCLC).[6] Nrf2 is associated with Kelch-like ECH-associated protein 1 (Keap1), its inhibitory protein, which is inactivated by reactive oxygen species (ROS) acting as a sensor. Oxidation of its 2 cysteines to yield a disulfide bond causes a conformational change in and inactivation of Keap1.[7] Being a ROS-generating agent, *tert*-butylhydroquinone (tBHQ) can inactivate Keap1 to activate Nrf2.[8] ROS are considered to be involved in the etiology of many inflammatory diseases including retinitis, other types of inflammation, diabetes mellitus, ischemic organ injury, and cancer.[9] Thus, safe activators of Nrf2 may become new chemotherapeutic agents. Therefore, we have begun screening for Nrf2 activators of

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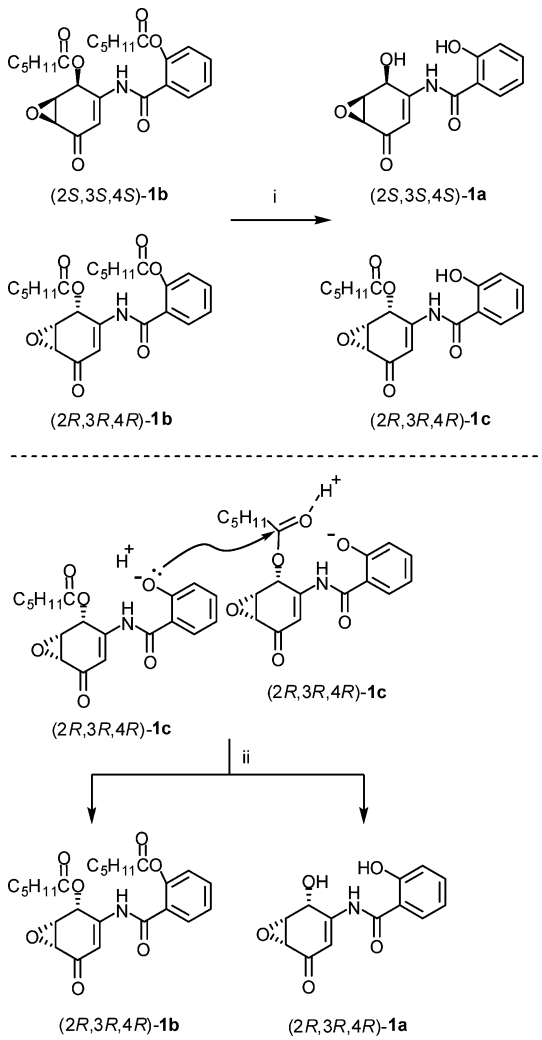
low-molecular weight by conducting the promoter reporter assay using luciferase. We employed microbial culture filtrates, plant extracts, and our own chemical library as the source of active compounds. Among hundreds of natural sources and chemicals, we found that (2*R*,3*R*,4*R*)-DHMEQ strongly activated the Nrf2 promoter activity in human neuroblastoma SK-N-SH cells.

Thus, efficient preparation of (2*R*,3*R*,4*R*)-DHMEQ should be useful to develop a new Nrf2 activator. In the present study we employed lipase again to prepare (2*R*,3*R*,4*R*)-DHMEQ, but with a completely different design for its synthesis. As a result, we succeeded in its effective synthesis by preparing the geometric isomer of the intermediate as the substrate of lipase.

Results and discussion

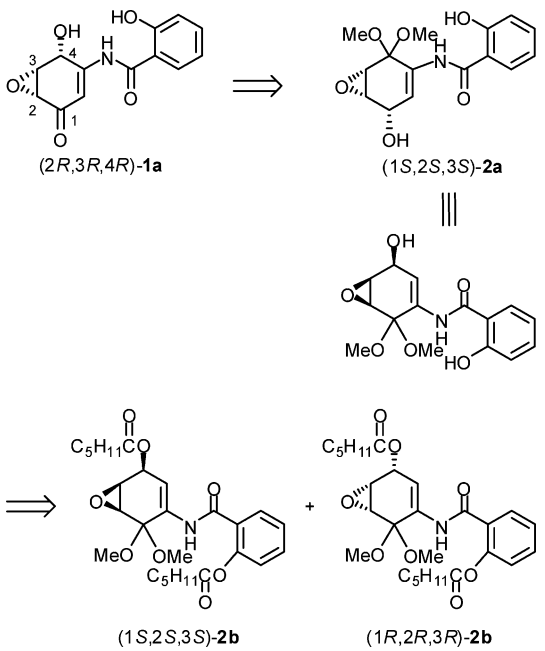
Chemoenzymatic synthesis of (2*R*,3*R*,4*R*)-DHMEQ

Previously reported enantiomeric resolution of (±)-**1a** by means of *Burkholderia cepacia* lipase-catalyzed enantio-selective hydrolysis of the corresponding dihexanoate **1b** is shown in Scheme 1.[5] The hydrolysis proceeds to give enantiomerically pure (2*S*,3*S*,4*S*)-**1a**, and the antipodal (2*R*,3*R*,4*R*)-isomer stays as its monoacylated form **1c**. The chromatographic separation of (2*S*,3*S*,4*S*)-**1a** and (2*R*,3*R*,4*R*)-**1c** has not been successful, due to the very unstable nature of this monoacylated form **1c** when in contact with silica gel. This undesired side reaction results in the disproportionation into **1a** and **1b**. So far, the only way to isolate the (2*S*,3*S*,4*S*)-isomer, the active form of the NF-κB inhibitor, has been the fractional crystallization of the mixture of **1a** and **1c**. This very simple protocol, the combination of kinetic resolution and fractional crystallization, cannot be applied to isolate the (2*R*,3*R*,4*R*)-isomer, because the latter always suffers contamination by the (2*S*,3*S*,4*S*)-isomer. A small amount of the (2*S*,3*S*,4*S*)-enantiomer always stays in the mother liquor as its diol after the crystallization.



Scheme 1 Reagents and conditions: (i) *Burkholderia cepacia* lipase (Amano PS-IM), acetone–water; (ii) disproportionation in silica gel column chromatography.

For the synthesis of (2*R*,3*R*,4*R*)-**1a**, our new approach is shown in Scheme 2. We chose an epoxy alcohol (1*S*,2*S*,3*S*)-**2a**, whose racemic form had been reported earlier,[1] as the precursor for **1a**. When this alcohol is displayed in an upside-down orientation, it appears to be similar to **1a**, and so we expected lipase-catalyzed kinetic resolution of the corresponding dihexanoate **2b**. We turned our attention to the acidic proton in the phenolic hydroxyl group in the salicylamide moiety in **1c**, which might cause an activation of the carbonyl group in the secondary ester group, to induce the undesired disproportionation, due to an intermolecular acyl migration (Scheme 1). An enhanced stability was expected for the monohexanoate **2c** compared with **1c** by shifting the salicylamide moiety to a little more remote position.



Scheme 2 Plan for the synthesis of (2*R*,3*R*,4*R*)-**1a**.

We were interested in the enantiomeric preference between **1b** and **2b**, by changing the position of the side chain from close to remote. In many cases, the enantio-selectivity depends upon the difference in the sizes between the substituents that are attached to the secondary acyloxy carbon. In the so far proposed empirical rules, when a large substituent is placed on the right and a small one is on the left, the hydrolysis of the isomer whose acyloxy group is forward is faster than that where the acyloxy group is backward. At this stage, we were not able to predict whether

the (1*R*,2*R*,3*R*)- or (1*S*,2*S*,3*S*)-isomer would be preferentially hydrolyzed by the lipase. Controversial results have been reported so far, as shown in Fig. 2. The (5*S*)-stereoisomer in (±)-**3** is the "fast" reacting isomer for lipase,[10] while in the case of (±)-**4**, the (1*R*)-stereoisomer is preferred.[11] In the former case, lipase recognized the methoxycarbonyl group as the large substituent and the epoxy ring as the small substituent, however, on the contrary, in the latter case the epoxy ring was larger than the methyl group.

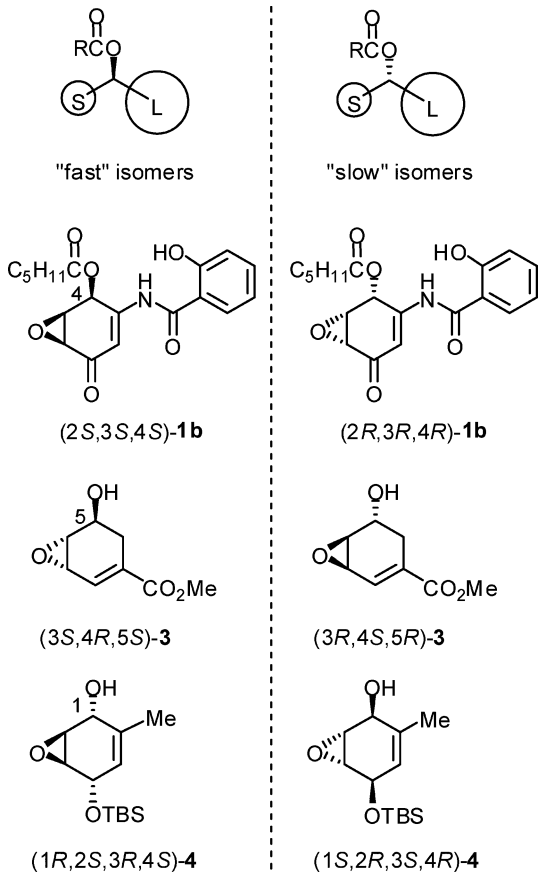
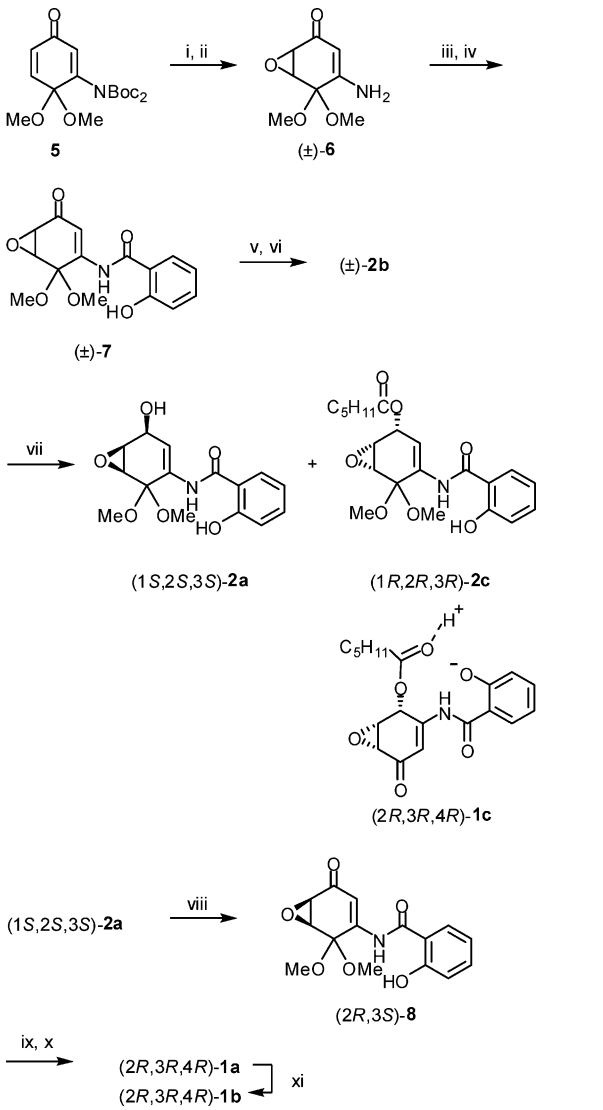


Fig. 2 "Fast" and "slow" reacting stereoisomers of epoxy ester **1b** and alcohols **3** and **4** in the action by lipase-catalyzed kinetic enantiomeric resolution.

Toward this end, dienone **5**[5] was treated with alkaline hydrogen peroxide solution (Scheme 3). As had been experienced in the asymmetric epoxidation, the complete blocking of the amino substituent with two Boc groups to exclude any acidic NH proton was quite advantageous for a high yield and reproducibility. The subsequent treatment of acidic deprotection of the Boc groups provided (±)-**6** in 80% yield for the two steps. The sequential treatment of **6** with the condensation between acetylsalicyloyl chloride and then deacetylation provided the known (±)-**7**[1a] in 65% yield for the two steps. The epoxyenone was derivatized to become the enzyme substrate (±)-**2b**, by Taylor's *syn*-stereoselective reduction[12] and the subsequent hexanoylation of the two free hydroxy groups in 89% yield for the two steps.

When (±)-dihexanoate **2b** was treated with *Burkholderia cepacia* lipase (Amano PS-IM) in phosphate buffer solution (pH 7.0), first the phenolic hexanoyl group in both enantiomers was



Scheme 3 Reagents and conditions: (i) H_2O_2, aq. NaOH; (ii) TFA, anisole, room temp. (80% for the 2 steps); (iii) *tert*-BuOLi, acetylsalicyloyl chloride, THF, −78 °C; (iv) K_2CO_3, MeOH, room temp. (65% for the 2 steps); (v) $LiBHEt_3$, THF, room temp.; (vi) hexanoic anhydride, DMAP, room temp. (89% for the 2 steps); (vii) *Burkholderia cepacia* lipase (Amano PS-IM), acetone–buffer (pH 7.0); (viii), MnO_2, CH_2Cl_2, room temp. (quantitative); (ix) TFA, 40 °C; (x) $NaBH(OAc)_3$, MeOH, 0 °C (87% for the 2 steps); (xi) hexanoic anhydride, DMAP, room temp.

promptly hydrolyzed to **2c**. This reaction proceeded in a non-enantioselective manner, similar to that observed in the lipase-catalyzed hydrolysis from **1a** to **1c**. The second step, the hydrolysis of the secondary hexanoate, was highly enantio-selective. As expected, the monohexanote **2c** was quite stable, even when in contact with the silica gel, in contrast to **1c**, which was susceptible to intramolecular hydrogen bonding between the acyl carbonyl group and acidic phenolic proton. Thus we were successful in separating completely **2a** and **2c** by column chromatography. The ees of **2a** and **2c** were estimated to be >99.9% and 66.2%, respectively, (see Experimental), and the *E* value (enantiomeric ratio)[13] was calculated to be over 500. The conversion was 40%, and the hydrolysate ("fast" reacting isomer) was obtained in an enantiomerically pure state.

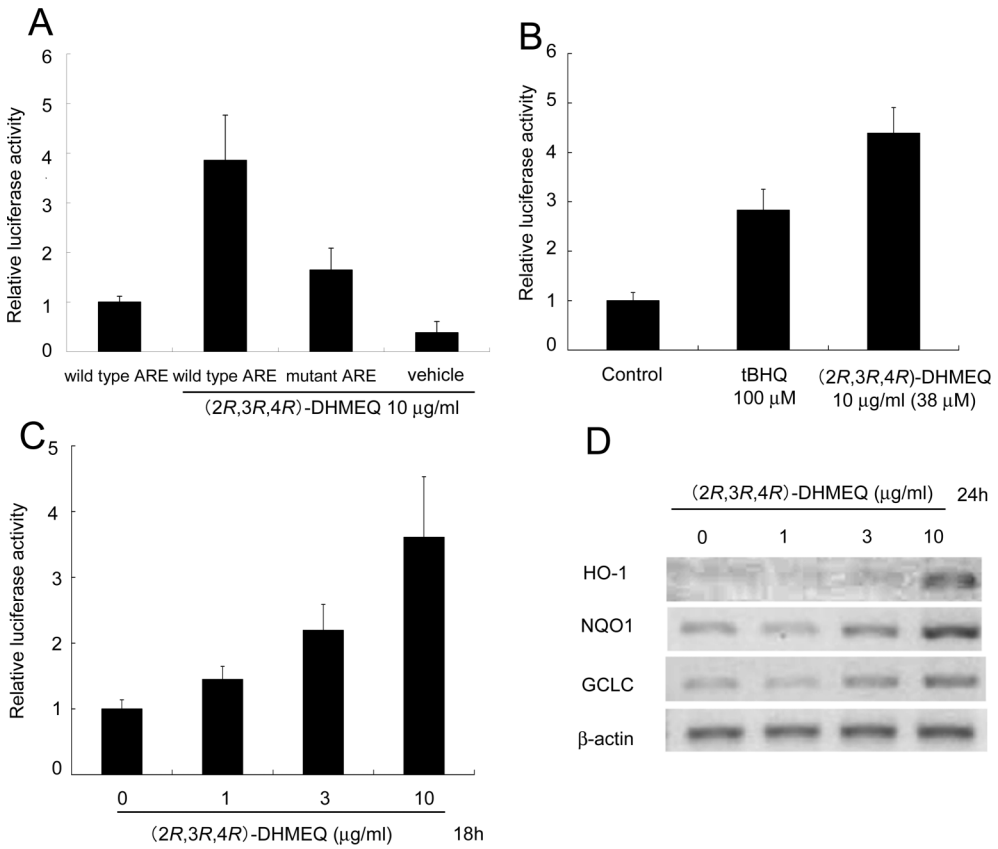


Fig. 3 Activation of Nrf2 in human neuroblastoma SK-N-SH cells. (A) (2*R*,3*R*,4*R*)-DHMEQ activated Nrf2 in the luciferase reporter assay only with the wild-type ARE but not with mutant ARE. (B) Comparison of the activity with tBHQ, a known activator of Nrf2. (C) Dose-responsive activation of Nrf2 by (2*R*,3*R*,4*R*)-DHMEQ in the reporter assay. (D) Enhancement of Nrf-2-dependent antioxidant enzyme expression by (2*R*,3*R*,4*R*)-DHMEQ. The mRNA levels were measured by RT-PCR.

The "fast" reacting isomer, (–)-**2a** was converted to DHMEQ (**1a**). First, MnO_2-mediated oxidation of the allylic alcohol provided (+)-**8**. As the epoxy ring in **2a** seemed to be sensitive to acid or base, and moreover, because a free phenolic hydroxy group was involved, we chose as mild conditions as possible. Deprotection of the dimethyl acetal under treatment with trifluoroacetic acid and the subsequent regio- and diastereo-selective reduction[1a] yielded (2*R*,3*R*,4*R*)-DHMEQ **1a**. Its enantiomeric purity was confirmed by the HPLC analysis (CHIRALCEL® AD-H) of the corresponding dihexanoate **1b**. The absolute configuration of (–)-**2a** was unambiguously determined to be (1*S*,2*S*,3*S*), and the enantiomeric preference between **1b** and **2b** was proved to be the same, in the lipase-catalyzed recognition. The plentiful supply of (2*R*,3*R*,4*R*)-DHMEQ **1a** was thus established in this way, by this newly designed route.

Activation of Nrf2 by (2*R*,3*R*,4*R*)-DHMEQ

As shown in Fig. 3A, (2*R*,3*R*,4*R*)-DHMEQ clearly induced activation of Nrf2 with the wild-type binding site (antioxidant responsive element, ARE), but not with the mutant ARE. Its activity was stronger than that of tBHQ, which is often used as the positive control (Fig. 3B). (2*R*,3*R*,4*R*)-DHMEQ activated Nrf2 dose dependently at 1–10 μg mL^{-1} (Fig. 3C). HO-1, NQO1, and GCLC are Nrf2-dependent antioxidant enzymes. The expression of their mRNA was also increased by the addition of (2*R*,3*R*,4*R*)-DHMEQ to SK-N-SH cell cultures (Fig. 3D). (2*R*,3*R*,4*R*)-DHMEQ did not show any cellular cytotoxicity at 10 μg mL^{-1}. Although (2*R*,3*R*,4*R*)- DHMEQ increased antioxidant enzyme expression, it did not change the expression of Nrf2 or Keap1. Instead, it enhanced the nuclear translocation of Nrf2 (data not shown).

ROS often induces the death of neurons, and thus may be involved in the etiology of neurodegenerative diseases. ROS-generating 6-hydroxydopamine hydrochloride (6-OHDA) induced the death of SK-N-SH cells, as shown in Fig. 4, and (2*R*,3*R*,4*R*)-DHMEQ decreased the cytotoxic effect of 6-OHDA, whereas its effect was weaker in Nrf2-knockdown cells prepared with siRNA. Thus, enhancement of the neural cell viability by (2*R*,3*R*,4*R*)-DHMEQ should be due to the activation of Nrf2.

Inhibition of NF-κB has been reported to activate the Nrf2 activity.[14] (2*S*,3*S*,4*S*)-DHMEQ also showed similar Nrf2 activating activity, while commercially available NF-κB inhibitors, BAY11-7082 and 1-pyrrolidine carbodithioic acid (PDTC), showed weaker activity. Since (2*R*,3*R*,4*R*)-DHMEQ possess only a weak NF-κB inhibitory activity, it would be unlikely that the activation is due to its inhibition of NF-κB. The mechanism of activation remains to be studied. In addition to tBHQ, known Nrf2 activators include sulforaphane[15] isolated from broccoli and carnosic acid[16] isolated from rosemary, however, no activator has been developed into the chemotherapeutic agent. Since (2*S*,3*S*,4*S*)-DHMEQ shows very little toxicity in animals, it is

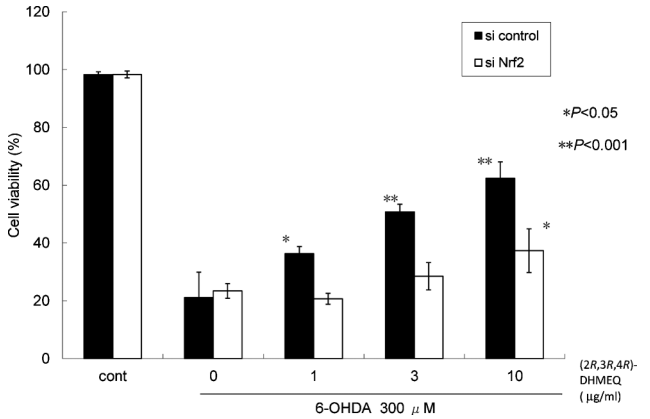


Fig. 4 Inhibition of 6-OHDA-induced death of SK-N-SH cells by (2*R*,3*R*,4*R*)-DHMEQ. The cells were treated with Nrf2 siRNA (white) or the control siRNA (dark), and then the chemicals were added. The cell viability was assayed by Trypan blue dye exclusion. For the statistical analysis, each value was compared with the value without (2*R*,3*R*,4*R*)-DHMEQ. *P<0.05; **P<0.001.

likely that (2*R*,3*R*,4*R*)-DHMEQ would also be nontoxic. It may be a new candidate for anti-inflammatory and anticancer agents.

Conclusions

By developing a newly designed substrate demonstrating high enantioselectivity (E >500), both enantiomeric precursors that led to (2*R*,3*R*,4*R*)- or (2*S*,3*S*,4*S*)-DHMEQ became accessible by applying lipase-catalyzed hydrolysis. This methodology is especially useful for the large-scale preparation of (2*R*,3*R*,4*R*)-DHMEQ, whose availability was previously very poor. In the course of our screening for Nrf2 activators, (2*R*,3*R*,4*R*)-DHMEQ was newly found to activate the promoter activity of the antioxidant transcription factor Nrf2. It also induced the expression of many antioxidant enzymes and canceled ROS-induced cell death in a neuronal cell line. Since the activity of (2*R*,3*R*,4*R*)-DHMEQ may not depend on conventional NF-κB inhibition, it may be a candidate for a new chemotherapeutic agent for inflammation and cancer.

Experimental

Chemistry—general methods

IR spectra were measured as thin films for oils or ATR for solids on a Jeol FT-IR SPX60 spectrometer. ^{1}H NMR and ^{13}C NMR spectra were measured in $CDCl_3$ at 400 MHz and 100 MHz on a VARIAN 400-MR spectrometer. HPLC data were recorded with a SHIMADZU SPD-M20A multi-channel detector. Optical rotation values were recorded by a Jasco P-1010 polarimeter. Silica gel 60 N (spherical, neutral, 63–210 μm, 37565–79) from Kanto Chemical Co. was used for column chromatography. Preparative TLC was performed with Merck Silica Gel 60 F_{254} plates (0.5 mm thickness, No. 1.05744).

(2*R,3*S**)-5-Amino-2,3-epoxy-4,4-dimethoxycyclohex-5-en-1-one (6).** To a solution of 3-*N*,*N*-bis(*tert*-butoxycarbonyl)amino-4,4-dimethoxycyclohexa-2,5-dien-1-one (**5**, 1.00 g, 2.71 mmol) in THF (30 mL) was added dropwise 29% aqueous H_2O_2 (29%, 5.4 mL, 43.2 mmol) and NaOH aq. solution (1 M, 10.8 mL, 10.8 mmol) at 0 °C. The mixture was stirred for 20 min and then allowed to warm to room temperature. The mixture was quenched with the addition of MnO_2, and filtered through a pad of Celite to remove insoluble materials. The filtrate was neutralized with saturated NH_4Cl aq. solution and then extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 (10 mL), and the solution was treated with trifluoroacetic acid (2.5 mL, 2.70 mmol) and anisole (1.0 mL) at room temperature[17] to give (2*R**,3*S**)-**6** (400.0 mg, 80% for the 2 steps) as a colorless oil. Its IR and ^{1}H NMR spectrum were identical with those reported previously.[17]

(2*R,3*S**)-2,3-Epoxy-5-*N*-[(2-hydroxybenzoyl)amino]-4,4-dimethoxycyclohex-5-en-1-one (7).** According to the reported procedure,[5,18] the above-mentioned enamine [(±)-**6**, 575.0 mg, 3.11 mmol] afforded **7** (615.0 mg, 65% for the 2 steps) as a colorless oil. Its IR and ^{1}H NMR spectra were identical with those reported previously.[1a]

(1*R,2*R**,3*R**)-2,3-Epoxy-5-*N*-[(2-hexanoylbenzoyl)amino]-4,4-dimethoxycyclohex-5-enyl hexanoate (2b).** According to the reported procedure,[12] to a solution of **7** (500 mg, 1.6 mmol) in anhydrous THF (2 mL) was added $LiBHEt_3$, (1 M in THF, KANTO CHEMICAL Inc. Japan, 24157–25, 3.2 mL, 3.2 mmol) in a portion-wise manner. After stirring for 1 h at −78 °C, the mixture was quenched with H_2O (5.0 mL), and then extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*.

Then, the residue was diluted in pyridine (4 mL), and to the mixture were added hexanoic anhydride (0.90 mL, 3.90 mmol) and DMAP (79.3 mg). After having been stirred for 1.5 h at room temperature, the mixture was quenched with H_2O (2.0 mL) and then extracted with EtOAc. The organic layer was washed with saturated NH_4Cl aq. solution and brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo.* The residue was purified by silica gel column chromatography (40 g). Elution with hexane/EtOAc = 10 : 1 afforded **2b** (717.7 mg, 89% for the 2 steps) as a colorless oil. ^{1}H NMR δ (ppm); 0.88 (t, J = 6.9 Hz, 3H), 0.91 (t, J = 7.1 Hz, 3H), 1.31 (m, 2H), 1.31 (m, 2H), 1.37 (m, 2H), 1.37 (m, 2H), 1.66 (m, 2H), 1.73 (m, 2H), 2.38 (t, J = 7.6 Hz, 2H), 2.62 (t, J = 7.6 Hz, 2H), 3.23 (s, 3H), 3.55, (d, J = 4.6 Hz, 1H), 3.56 (s, 3H), 3.63 (ddd, J = 2.4, 2.4, 4.6 Hz, 1H), 5.87 (dd, J = 2.4, 2.4 Hz, 1H), 6.68 (dd, J = 2.4, 2.4 Hz, 1H), 7.09 (dd, J = 1.0, 7.8 Hz, 1H), 7.30 (ddd, J = 1.0, 7.5, 7.8 Hz, 1H), 7.47 (ddd, J = 1.4, 7.5, 7.8 Hz, 1H), 7.83 (dd, J = 1.4, 7.8 Hz, 1H), 8.38 (s, 1H); ^{13}C NMR δ (ppm) 13.8, 13.9, 22.2, 22.3, 24.3, 24.5, 31.2, 31.3, 34.0, 34.2, 50.2, 50.5, 51.0, 67.1, 77.2, 95.7, 110.5, 123.4, 126.2, 127.8, 129.7, 130.2, 132.3, 147.9, 163.6, 171.8, 173.4, IR ν_{max} 3398, 2954, 2931, 2870, 1767, 1736, 1678, 1608, 1512, 1342, 1199, 1161, 1084, 1057, 976, 937, 906 cm^{-1}. Anal. Calcd for $C_{27}H_{37}NO_8$: C, 64.40; H, 7.41; N, 2.78. Found: C, 64.39; H, 7.42; N, 3.47. Analysis was performed by HPLC [CHIRALCEL® AD-H, 0.46 cm × 25 cm; hexane-2-propanol (5 : 1); flow rate 0.5 mL min^{-1}; detected at 260 nm]: t_R (min) = 23.5 (50.2%), 26.7 (49.8%).

***Burkholderia cepacia* lipase-catalyzed hydrolysis of (1*R**,2*R**,3*R**)-2b.** To a solution of (1*S*,2*S*,3*S*)-**2b** (450.0 mg, 0.89 mmol) in acetone (6.0 mL) and phosphate buffer

solution (pH 7.0, 0.1 M, 6.0 mL) was added *Burkholderia cepacia* lipase (Amano PS-IM, 615 mg). After vigorous stirring for 48 h at 30 °C, the mixture was filtered through a pad of Celite to remove insoluble materials, and then extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo.* The residue was purified by silica gel column chromatography (30 g). Elution with hexane/EtOAc = 4 : 1 afforded (1*S*,2*S*,3*S*)-2,3-epoxy-5-*N*-[(2-hydroxybenzoyl)amino]-4,4-dimethoxycyclohex-5-en-1-ol (**2a**, 110.0 mg, 40%) and (1*R*,2*R*,3*R*)-2,3-epoxy-5-*N*-[(2-hydroxybenzoyl)amino]-4,4-dimethoxycyclohex-5-enyl hexanoate (**2c**, 211.0 mg) as a colorless oil.

(1*S*,2*S*,3*S*)-**2a**: $[\alpha]_D^{25}$ −3.2 (*c* 1.00, $CHCl_3$); ^{1}H NMR δ (ppm): 3.25 (s, 3H), 3.62 (s, 3H), 3.62 (s, 1H), 3.62 (s, 1H), 4.73 (brs, 1H), 6.66 (s, 1H), 6.87 (dd, *J* = 7.2, 8.0 Hz, 1H), 6.98 (d, *J* = 8.0 Hz,1H), 7.31 (d, *J* = 7.0 Hz, 1H), 7.41 (dd, *J* = 7.0, 7.2 Hz, 1H), 8.21 (s, 1H), 11.9 (s, 1H); ^{13}C NMR δ (ppm): 50.4, 50.9, 51.6, 53.1, 65.1, 95.6, 114.7, 115.6, 118.9, 119.0, 125.2, 127.8, 134.7, 161.7, 168.4; IR ν_{max} 3410, 3298, 2981, 2943, 2835, 1736, 1647, 1527, 1450, 1354, 1234, 1126, 1041, 937, 906, 756 cm^{-1}. Anal. Calcd for $C_{15}H_{17}NO_6$: C, 58.63; H, 5.58; N, 4.56. Found: C, 58.81; H, 6.19; N, 3.79. HPLC [CHIRALCEL® AD-H, 0.46 cm × 25 cm; hexane-2-propanol (3 : 1); flow rate 0.5 mL min^{-1}; detected at 290 nm]: t_R (min) = 14.6 (single peak). The separation of enantiomers was confirmed by the analysis of its racemate, t_R (min) = 12.4 (49.8%), 14.6 (50.2%).

(1*R*,2*R*,3*R*)-**2c**: ^{1}H NMR δ (ppm); 0.88 (t, *J* = 7.0 Hz, 3H), 1.31 (m, 2H), 1.31 (m, 2H), 1.66 (m, 2H), 2.39 (t, *J* = 7.6 Hz, 2H), 3.26 (s, 3H), 3.58, (d, *J* = 4.5 Hz, 1H), 3.62 (s, 3H), 3.63 (ddd, *J* = 2.3, 2.4, 4.5 Hz, 1H), 5.87 (dd, *J* = 2.2, 2.4 Hz, 1H), 6.56 (dd, *J* = 2.2, 2.3 Hz, 1H), 6.87 (ddd, *J* = 1.0, 7.2, 8.2 Hz, 1H), 6.97 (dd, *J* = 1.0, 8.2 Hz, 1H), 7.30 (dd, *J* = 1.5, 7.2 Hz, 1H), 7.40 (ddd, *J* = 1.5, 8.2, 8.2 Hz, 1H), 8.30 (s, 1H), 11.8 (s, 1H). Its ee was confirmed by derivation to the corresponding dihexanoate (1*R*,2*R*,3*R*)-**2b**. HPLC analysis was performed in the same manner as above: t_R (min) = 23.5 (83.1%), 26.7 (16.9%).

(2*R*,3*S*)-2,3-Epoxy-5-*N*-[(2-hydroxybenzoyl)amino]-4,4-dimethoxycyclohex-5-en-1-one (8). To a solution of **2a** (128.0 mg, 0.42 mmol) in CH_2Cl_2 was added activated MnO_2 (Eastman Kodak Co., 50199, 185 mg). After having been stirred for 24 h at room temperature, the mixture was filtered through a pad of Celite to remove insoluble materials. The residue was washed with EtOAc. The combined filtrate and washings were concentrated *in vacuo* to afford **8** (124.8 mg, 98%) as a yellow oil; $[\alpha]_D^{24}$ +200 (*c* 1.00, $CHCl_3$). Its IR and ^{1}H NMR spectra were identical with those reported previously.[3] This compound was employed for the next step without further purification.

(2*R*,3*R*,4*R*)-2,3-Epoxy-4-hydroxy-5-*N*-[(2-hydroxybenzyl)-amino]cyclohex-5-en-1-one (1a). A solution of **8** (118.0 mg, 0.39 mmol) in TFA (0.78 mL) was stirred for 1.5 h at 40 °C. The mixture was concentrated *in vacuo* to afford (2*R*,3*S*)-2,3-epoxy-5-*N*-[(2-hydroxybenzoyl)amino]cyclohex-5-ene-1,4-dione (99.9 mg, 99%) as a yellow solid; $[\alpha]_D^{24}$ +51.3 (*c* 1.00, MeOH). Its IR and ^{1}H NMR spectra were identical with those reported previously for the racemic form. This compound was employed for the next step without further purification.

According to the reported procedure,[1,3] this diketone (99.9 mg, 0.39 mmol) was reduced with $NaBH(OAc)_3$ in MeOH. After workup, the residue was purified by preparative TLC using MeOH : $CHCl_3$ (1: 10, v/v) as mobile phase to yield **1a** (88.6 mg, 88%) as a white solid. Mp 184 °C [lit.[1b] mp 185 °C]. Its ee and the absolute configuration were confirmed after derivation to dihexanoate (2*R*,3*R*,4*R*)-**1b**. Colorless oil; $[\alpha]_D^{26}$ +114 (*c* 0.68, $CHCl_3$) [lit.[5] $[\alpha]_D^{23}$ −126 (*c* 0.66, $CHCl_3$) for (2*S*,3*S*,4*S*)-**1b**]. Its IR and ^{1}H NMR spectra were identical with those reported previously. HPLC [CHIRALCEL® AD-H, 0.46 cm × 25 cm; hexane-2-propanol (7 : 1); flow rate 0.5 mL min^{-1}; detected at 290 nm]: t_R (min) = 28.3 (single peak). There was no peak at 30.9 min ascribable to (2*S*,3*S*,4*S*)-**1b**.

Biological assays

Materials

6-OHDA was purchased from Sigma (St. Louis, MO). Human neuroblastoma SK-N-SH cells were obtained from Riken Bio-Resource Center (Tsukuba, Japan).

Cell culture

SK-N-SH cells were grown in Minimum Essential Medium Alpha (MEMα, Invitrogen Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Nichirei Biosciences, Tokyo, Japan), 200 μg mL^{-1} kanamycin, 100 U mL^{-1} penicillin G, 600 μg mL^{-1} L-glutamine, and 2.2 g L^{-1} $NaHCO_3$.

Plasmid construction and reporter luciferase assays

The human wild-type (5′-GTGACTCAGCA-3′) and mutant (5′-GCGACTCAGCA-3′) ARE sequences of human NQO1 were cloned upstream of the luciferase reporter gene by using sense and antisense oligonucleotides synthesized to have *Bgl*II and *Xho*I sites, respectively. The oligonucleotides were annealed, phosphorylated using T4 polynucleotide kinase, and cloned into the respective sites in the pGL4.17 firefly luciferase reporter vector (Promega, Madison, WI). Then, SK-N-SH cells were transfected with wild-type or mutant ARE sequence vector, or empty vectors, and phRL TK vectors (Promega) using lipofectamine™ LTX Reagent (Invitrogen). After a 24 h incubation at 37 °C, the cells were treated with chemicals, and then 18 h later the luciferase activity was measured. The results were normalized with the Renilla luciferase activity.

Reverse transcription (RT)-PCR

SK-N-SH cells were grown to confluence in 60 mm dishes and treated with (2*R*,3*R*,4*R*)-**1a**. Total RNA was prepared from the cells by using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). The following primers were used for PCR: HO-1 Forward, 5′-ATGACACCAAGGACCAGAGC-3′; HO-1 Reverse, 5′-GTGTAAGGACCCATCGGAGA-3′; NQO1 Forward, 5′-CACTGATCGTACTGGCTCACTC-3′; NQO1 Reverse, 5′- AAGGGTCCTTTGTCATACATGG-3′; GCLC Forward, 5′-CCCATGGAGGTGCAATTAAC-3′; GCLC Reverse, 5′-TGCGATAAACTCCCTCATCC-3′; β-actin Forward, 5′-TTCTACAATGAGCTGCGTGT-3′; β-actin Reverse, 5′-GTCAGGTCCCGGCCAGCCAG-3′.

Knockdown of Nrf2 siRNA

Transfection of cells with siRNA was performed by using the Xtreme Gene reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Briefly, the cells were incubated overnight at 37 °C in 500 μL of complete medium in each well of a 24-well plate. The next day, the medium in each well was changed to opti-MEM. The transfection reagent was prepared as follows: 10 μL of Xtreme transfection reagent was mixed with 90 μL of opti-MEM (Invitrogen) in a 1.5 mL tube, and the desired volume of siRNA for Nrf2 (5′- UrCrCrCrGUUUrGUrArGrAUrGrArCrArATT -3′) (Sigma-Aldrich Japan, Tokyo, Japan) or control (5′- rCrArArGrCUrGrArCrCrCUrGrArArGUUr -3′) was mixed with 100 μL of opti-MEM in another 1.5 mL tube. These reagents were mixed and incubated at room temperature for 20 min. The mixed siRNA complex was added dropwise onto the cells, which were then incubated for 24 h.

Trypan blue dye exclusion

SK-N-SH cells were seeded at 1.75×10^4 cells/well in a 24-well plate (Costar) and cultured overnight. The cells were treated with various concentrations of (2*R*,3*R*,4*R*)-**1a** for 24 h and subsequently treated with 300 μM 6-OHDA for 24 h. Then, cells were stained with Trypan blue, and the number of stained cells was counted.

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