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In Vitro Biotransformation of (*R*)- and (*S*)-Thalidomide: Application of Circular Dichroism Spectroscopy to the Stereochemical Characterization of the Hydroxylated Metabolites

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Circular dichroism (CD) spectroscopy was successfully used for the stereochemical characterization of the hydroxylated metabolites formed during the in vitro biotransformation of (*R*)- and (*S*)-thalidomide. Incubation extracts of the individual enantiomers were analyzed by HPLC on an achiral stationary phase combined with CD detection. The CD data of the almost enantiopure eluates of the metabolites were compared with the CD spectra quantum chemically calculated for the respective structures. The results allowed us a reliable determination of the absolute stereostructure for all of the metabolites. The chiral center of thalidomide is unaffected by the stereoselective biotransformation process. (*3′R,5′R*)-*trans*-5′-hydroxythalidomide is the main metabolite of (*R*)-thalidomide, which epimerizes spontaneously to give the more stable (*3′S,5′R*)-*cis* isomer. On the contrary, (*S*)-thalidomide is preferentially metabolized by hydroxylation in the phthalimide moiety, resulting in the formation of (*S*)-5-hydroxythalidomide.

Thalidomide, identified to be a human teratogen in the early 1960s,[1,2] was recently approved for sale in the United States for the treatment of erythema nodosum leprosum, a complication of Hansen's disease.[3] The originally sedative−hypnotic drug was found to suppress the release of the cytokine tumor necrosis factor (TNF)-α and to inhibit angiogenesis.[4,5] Due to its antiinflammatory and immunomodulatory properties thalidomide has been used successfully in graft-versus-host disease, rheumatoid arthritis, and several dermatologic disorders, e.g., Behcet's syndrome and lupus erythematosus.[6,7] Notably, the enantiomers of thalidomide (Figure 1) show a significant enantioselectivity in pharmacodynamics. The immunomodulatory effects may chiefly be exerted by (*S*)-thalidomide,[8] whereas sedation was related to the blood concentration of the (*R*) enantiomer.[9]

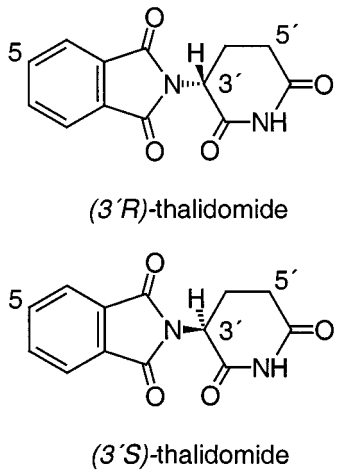

Figure 1. Structure of thalidomide enantiomers.

About four decades after Lenz[1] and McBride[2] independently associated the use of thalidomide in early pregnancy with the occurrence of phocomelia, the exact mechanism of teratogenesis remains unknown. Several different hypotheses were discussed, suggesting, for example, the bioactivation of thalidomide by embryonic prostaglandin H synthase, which causes oxidative damage to DNA.[10] The latest results proposed that the drug affects the pathway of insulin-like growth factor I and fibroblast growth factor 2 stimulation of α-v and *β*3 integrin subunit genes during development.[11]

Another tenable explanation of the teratogenic effects involves the metabolic formation of reactive arene oxide intermediates,

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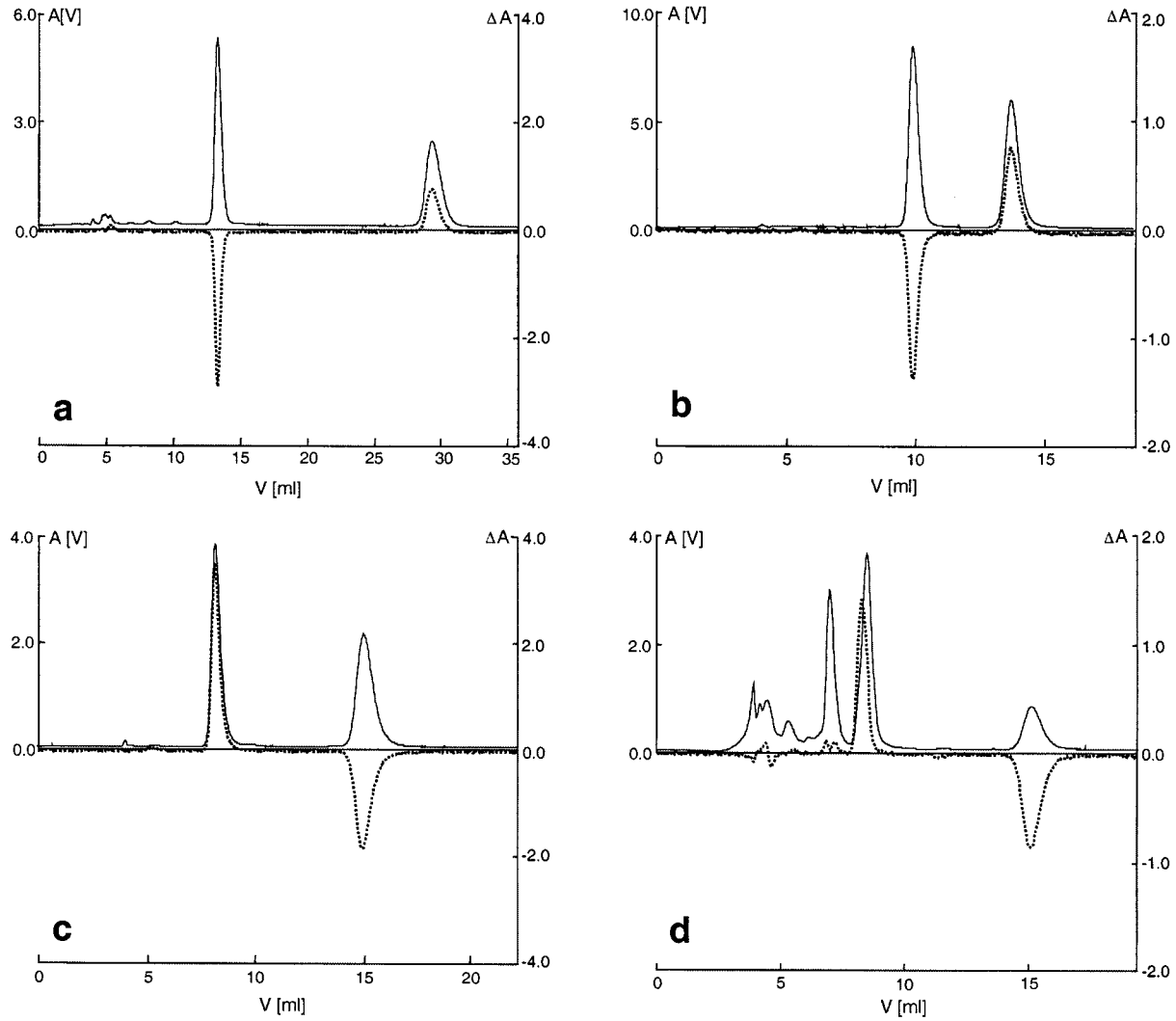

Figure 2. LC separation of the racemic reference compounds thalidomide (a), 5-hydroxythalidomide (b), *cis*-5′-hydroxythalidomide (c), and *trans*-5′-hydroxythalidomide (d) using circular dichroism ΔA (•••) and UV photometric (−) detection A at 240 nm. Column: Chiralpak AD 250 × 4.6 mm with a 30 × 4 mm precolumn. Eluent: MeOH. Flow rate: 1.5 (a), 0.7 (b), and 1.2 mL/min (c, d).

which are detoxified to (less reactive) phenols.[12] The application of thalidomide in an in vitro lymphocyte assay system provided the evidence that the drug itself was not toxic whereas toxicity required both a source of hepatic enzymes and a NADPH-generating system.[13] Although thalidomide was found to be a poor substrate for cytochrome P-450,[14] we have focused our work on the enzyme-catalyzed in vitro biotransformation by rat liver microsomes due to the observations mentioned above. The metabolites previously detected in these incubation extracts[15] were also found in plasma samples from male volunteers who had received thalidomide orally.[16]

Former studies using capillary electrophoresis with cyclodextrins as chiral selectors allowed the enantioselective separation of thalidomide and three hydroxylated derivatives.[15] These investigations demonstrated the stereoselective in vitro biotransformation: The (*R*) enantiomer is preferentially metabolized in the 2′,6′-dioxopiperidine-3′-yl moiety, whereas (*S*)-thalidomide is mainly transformed by hydroxylation in the phthalimide ring. On one hand, the enantiomers of 5-hydroxythalidomide and the diastereomeric 5′-hydroxythalidomides were detected in nonracemic ratios after 30-min incubation of the racemic drug. On the other hand, the metabolism of (*R*)-thalidomide was found to be highly product-enantioselective, resulting in the formation of one enantiomer of each metabolite exclusively. During the incubation of (*S*)-thalidomide, the corresponding optical antipodes of 5- and *trans*-5′-hydroxythalidomide were formed in high enantiomeric excesses of about 95% and 92%, respectively. The previous tentative assignments[15] of the absolute configuration of all metabolites are based on X-ray diffraction data, NMR experiments, configuration stability of the substrate, and enantioselective effects in biotransformation.

In the present study, we describe the detailed stereochemical characterization of the hydroxylated derivatives of thalidomide by means of experimental circular dichroism (CD) spectroscopy in combination with quantum chemical CD calculations. Another

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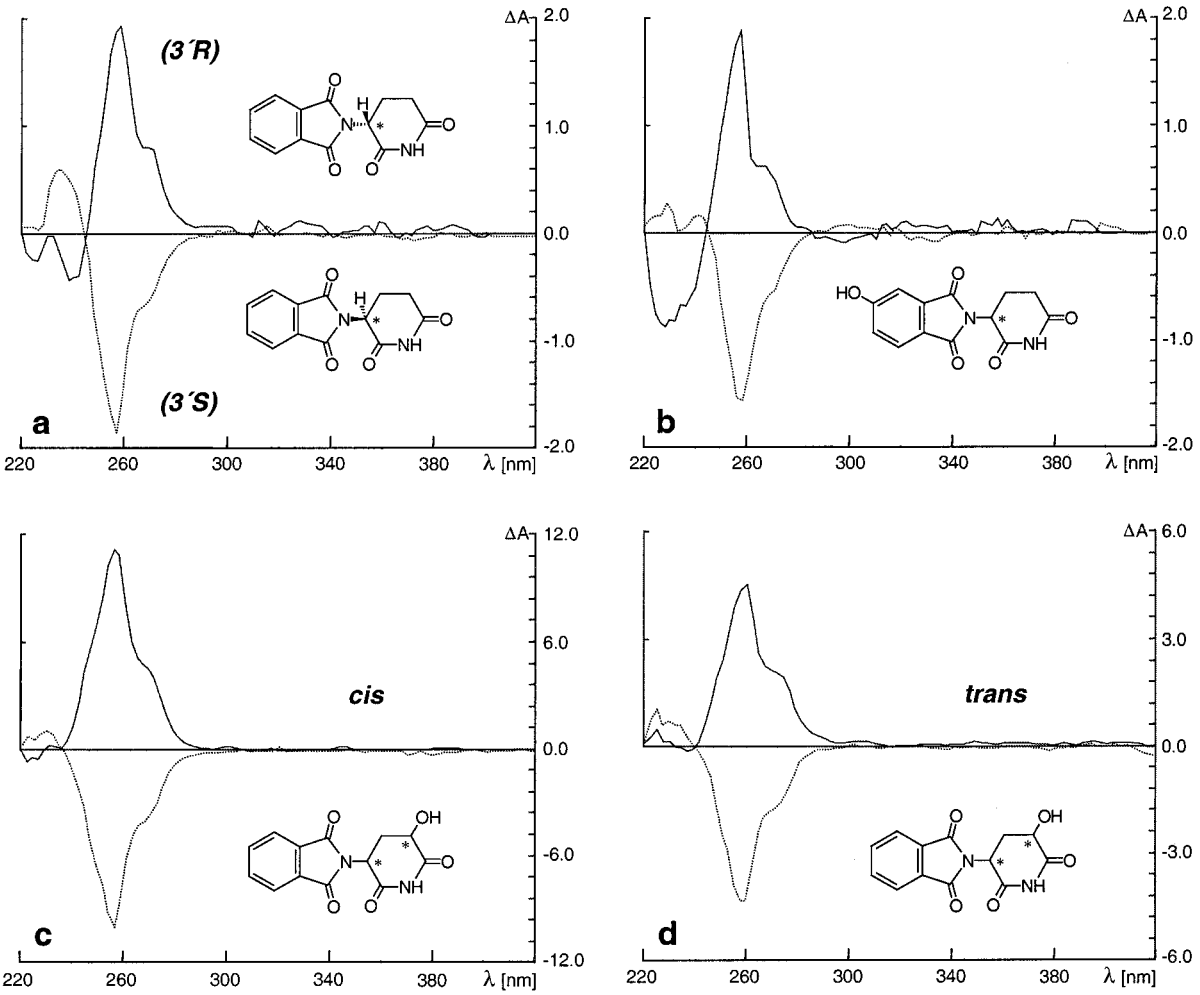

Figure 3. Stopped-flow aquisition of CD spectra (220−420 nm) of the racemic reference com pounds thalidomide (a), 5-hydroxythalidomide (b), *cis*-5′-hydroxythalidomide (c), and *trans*-5′-hydroxythalidomide (d) during LC, according to Figure 2. Chromatographic conditions as in Figure 2.

subject was to investigate the influence of the incubation time on the metabolic transformation, especially the epimerization of the thermodynamically less stable trans diastereomer of 5′-hydroxythalidomide to the corresponding cis isomer.

EXPERIMENTAL SECTION

Chemicals and Reagents. Racemic thalidomide (TD) was kindly provided by Grünenthal (Stolberg, Germany). The enantiomers of thalidomide were obtained in our laboratory by preparative low-pressure liquid chromatography on a poly(*S*)-*N*-(1-cyclohexylethyl)methacrylamide stationary phase.[17] The purity was determined by chiral HPLC and exceeded more than 99.5%. The racemic cis isomer of 5′-hydroxythalidomide (*cis*-5′-OH-TD) and the mixture of diastereomeric 5′-acetoxythalidomide pairs were gifts from Prof. K. Eger (University of Leipzig, Germany).[18] Racemic 5-hydroxythalidomide (5-OH-TD) was synthesized as described.[19] Sulfobutyl-β-cyclodextrin (SBE-β-CD) with an average molecular substitution degree of 4.0 was obtained from CyDex, L.C. (Overland Park, KS) and native β-cyclodextrin (β-CD) was kindly donated by Wacker Chemie (Munich, Germany). Analytical grade phosphoric acid, ammonium acetate, anhydrous methanol, acetonitrile, and glacial acetic acid were purchased from Merck (Darmstadt, Germany). Magnesium chloride and *p*-toluenesulfonic acid were from Aldrich (Daisenhofen, Germany). *N*-Acrylamide, *N*,*N*,*N*′,*N*′-tetramethylethyldiamine, (3-aminopropyl)triethoxysilane for the preparation of the coated capillaries, and reduced NADPH (tetrasodium salt) were supplied from Fluka (Fluka Chemie, Buchs, Switzerland). Methanol and ethyl acetate of HPLC quality were from J. T. Baker (Deventer, The Netherlands).

Circular Dichroism Spectroscopy. Chiroptical detection during liquid chromatography was performed using the Jasco CD-1595 detector (Jasco International Co., Ltd., Tokyo, Japan), which provides the differential absorbance ΔA of the CD and the absorbance A of the UV mode simultaneously as a function of time. The analog experimental data were monitored by the two-channel recorder Asea Brown Boveri SE 120 or digitized and processed using the computer program SEPP.[20,21] Simultaneous detection of the CD and the UV signals was carried out with a

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flow-through cell (25 mm) at the single wavelength of 240 nm. The spectra (220−420 nm) were recorded by a stopped-flow method[21] under the same conditions. Measurements were performed using a Merck-Hitachi L-6000A pump) equipped with a Rheodyne sample injector (Rheodyne, Cotati, CA) and an Erma ERC-3312 degasser (Erma CR Inc., Tokyo, Japan). The racemic reference compounds were separated on a Chiralpak AD (Daicel, Himeji, Japan) 250 × 4.6 mm column with a 30 × 4 mm precolumn using methanol as eluent. CD data of individual incubation extracts were obtained after chromatographic resolution on an achiral stationary phase as described below.

The CD spectra measured from 200 to 350 nm, as required for the comparison with the calculated spectra, were taken on a J-715 spectropolarimeter (Jasco GmbH Deutschland, Gross-Umstadt, Germany) at room temperature. The stand-alone spectra were measured in ethanol. The on-line spectra were measured in stopped-flow mode with a standard flow-through cell with methanol as the eluent.

Reversed-Phase HPLC. The HPLC system consisted of a Merck-Hitachi L-6200A pump equipped with a Rheodyne sample injector), a variable-wavelength 655A detector (Merck-Hitachi) used at 230 nm, and a D-2500 Chromato-integrator (Merck-Hitachi). The analytes of the incubation mixtures were separated on a 125 × 4 mm RP-18 stationary phase (LiChrospher 100 RP-18, 5 μm, Merck) with a 4 × 4 mm precolumn. The mobile phase was 1% acetic acid/acetonitrile (92:8, v/v), the flow rate 1.2 mL/min.

Capillary Electrophoresis. The HP[3D] capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector was used for the CE studies. Chiral separations were performed in capillaries of 50-cm effective length (EL) and 50-μm i.d. (Polymicro Technologies, Phoenix, AZ) which were previously coated with linear polyacrylamide as decribed.[22] A 12 mg/mL solution of β-cyclodextrin and 12 mg/mL SBE(4)-β-cyclodextrin as chiral selectors were dissolved in 5 mM ammonium acetate (BGE) at pH 4.5. The measurements were carried out with an applied voltage of −30 kV (reversed polarity), and the average current was 20 μA. Samples were injected hydrodynamically (50 mbar) for 7 s and detected at 230 nm. Between the analyses, the capillary was rinsed for 2 min with methanol and for another 2 min with run buffer.

Preparation of Liver Fractions and in Vitro Biotransformation by Hepatic Microsomes. Hepatic subcellular fractions were prepared from male Sprague−Dawley rats that had been pretreated with 50 mg/kg of body weight phenobarbital for 6 days. Livers were homogenized in buffer (25 mM sucrose/5 mM Tris-HCl/0.5 mM EDTA) at 4 °C. Supernatant (10000*g*) and microsomal fractions (100000*g*) were prepared according to the method described in ref 23 and stored at −80 °C.

The incubation mixture with a total volume of 1 mL consisted of substrate (20 μg of racemic thalidomide or 10 μg of each thalidomide enantiomer), 410 μL of 0.1 M Tris-buffer (pH 7.4 at 37 °C), 245 μL of NADPH solution (8 mg/mL buffer), 100 μL of magnesium chloride (0.06 M), and 245 μL of rat liver microsomal preparation. Incubations were carried out while stirring in a water bath at 37 °C. After different periods (5−120 min), the reaction was stopped by cooling to 0 °C. Thalidomide and its hydroxylated metabolites were extracted into 3 mL of ethyl acetate by shaking in a reciprocal shaker for 10 min. After centrifugation at 2500*g*, the organic layer was evaporated under a stream of nitrogen. The residue was dissolved either in the running buffer (CE) or in the mobile phase (HPLC) and analyzed.

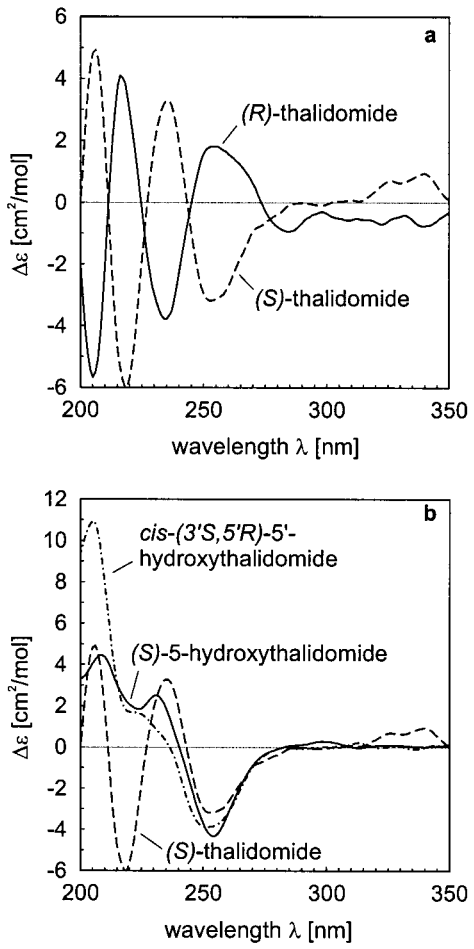

Figure 4. Stand-alone measured CD spectra of (*R*)- and (*S*)-thalidomide (a) and comparison of the experimental CD spectra featuring a negative CD band at 255 nm (b).

Hydrolysis of Reference Compounds. The mixture of diastereomeric 5′-acetoxythalidomide pairs was hydrolyzed according to ref 24. The compound was heated with *p*-toluenesulfonic acid in anhydrous methanol to give a mixture of both diastereomers of 5′-hydroxythalidomide. The individual isomers could be obtained by fractional separation using an achiral stationary phase.

Fractional Separation of Racemic *trans*-5'-Hydroxythalidomide. The thermodynamically less stable *trans*-5′-hydroxythalidomide was obtained by fractional separation on a 125 × 4 mm RP-18 (LiChrospher 100, 5 μm) column after hydrolysis of the mixture of diastereomeric 5′-acteoxythalidomide. The mobile phase was 1% acetic acid/acetonitrile (90:10, v/v) with a flow rate of 1.5 mL/min. The detection wavelength was 230 nm. The racemic compound was re-extracted from the mobile phase using ethyl acetate.

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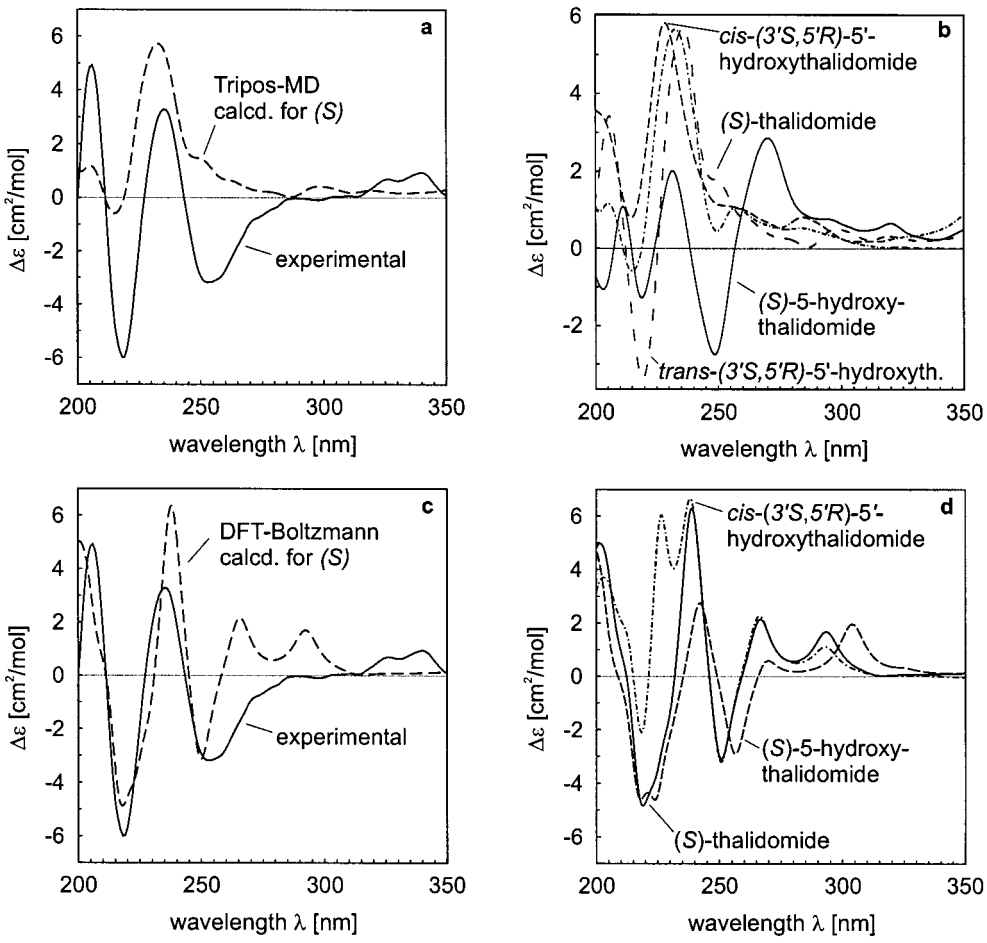

Figure 5. Comparisons of CD spectra: the calculated spectrum of (*S*)-thalidomide using the MD approach (- − -) with the experimental one (−) (a), the calculated spectra of all of the (*3′S*)-configured compounds using the MD approach (b), the experimental spectrum of (*S*)-thalidomide (−) with the one calculated using the DFT−Boltzmann approach (- − -) (c), and the spectra calculated for (*S*)-thalidomide (−), cis-*(3′S,5′R)*-5′-hydroxythalidomide (· − ·), and (*S*)-5-hydroxythalidomide (- − -) using the DFT−Boltzmann approach (d).

COMUPTATIONAL METHODS

Conformational Analysis. The conformational analyses of thalidomide, *cis*-5′-hydroxythalidomide, and 5-hydroxythalidomide were performed on Linux *i*PII and *i*PIII workstations by means of the DFT approach in the TZP[25] basis set employing the B3LYP functional.[26,27] All calculations were performed using the program package Turbomole[28] starting from preoptimized geometries generated by the AM1[29] parametrization as implemented in the program package VAMP.[30]

Molecular Dynamics (MD). The MD simulations were performed on Silicon Graphics Octane (R10000) workstations using the Tripos[31] force field as implemented in the molecular modeling package Sybyl.[31] The molecules were weakly coupled to a virtual thermal bath at $T = 700$ K,[32] with a temperature relaxation time $\tau = 0.1$ ps.

CD Calculations. The wave functions for the calculation of the rotational strengths for the electronic transitions from the ground state to excited states were obtained by CNDO/S-CI[33,34] calculations, in which the CI expansion[33,34] takes into account the ground state and all n and π orbitals. These calculations were carried out on Linux *i*PII and *i*PIII workstations using the BDZDO/MCDSPD[33] program package. For a better comparison of the theoretical CD spectra with the experimental ones, Gaussian band shape functions were generated over the calculated rotational strength values.

RESULTS AND DISCUSSION

Circular Dichroism of Authentic Standards. Molecules containing one or more centers of dissymmetry can be effectively studied using the various techniques of chiroptical spectroscopy,

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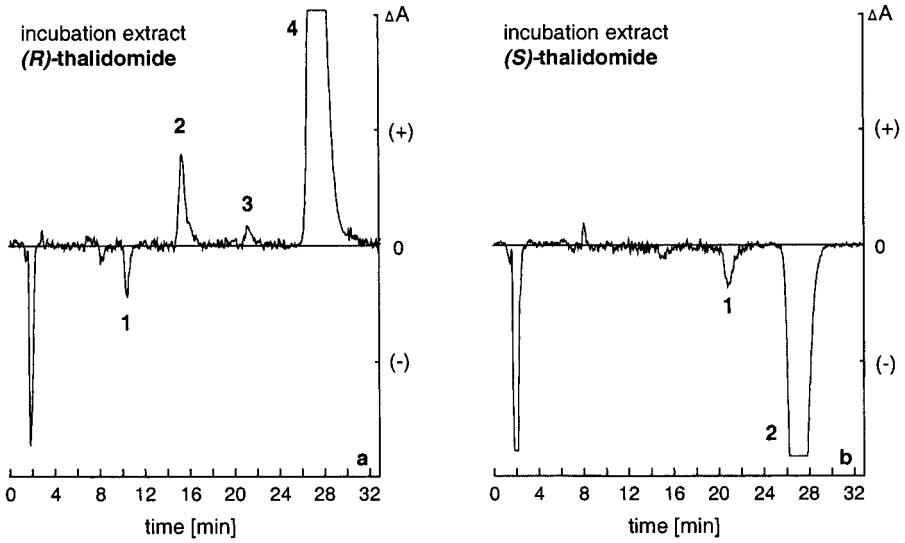

Figure 6. CD detection (250 nm) during LC of (*R*)-thalidomide (a) and (*S*)-thalidomide (b) and its hydroxylated metabolites, extracted from incubations with rat liver microsomes. Column: LiChrospher 100 RP-18, 125 × 4 mm with a 4 × 4 mm precolumn. Eluent: 1% acetic acid/acetonitrile (92:8, v/v). Flow rate: 1.2 mL/min. Peak assignments: (a) Single enantiomers of *cis*-5′-OH-TD (1), *trans*-5′-OH-TD (2), 5-OH-TD (3), and TD (4); (b) single enantiomers of 5-OH-TD (1) and TD (2).

e.g., circular dichroism.[35] Due to different absorption indexes of left- and right-circularly polarized beams in the optically active medium, this method has been successfully used for the detailed stereochemical characterization of enantiomeric eluates.[36,37]

The aim of the present work was to determine the absolute configuration of the hydroxylated metabolites of thalidomide by quantum chemical calculation of their circular dichroism spectra and comparison with the experimentally measured ones. For this reason, chiroptical detection during liquid chromatography was applied to monitor the differential absorbance ΔA of the four racemic standards thalidomide, 5-hydroxythalidomide, and the diastereomers of 5′-hydroxythalidomide. This procedure for the aquisition of the CD spectra does not need any preparative enrichment of enantiomers. Since a previous study illustrated the excellent enantioselectivity but insufficient chemoselectivity of a Chiralpak AD column for this particular set of analytes,[38] the above racemates were separated individually on this optically active sorbent using methanol as eluent (Figure 2). Separation characteristics calculated by the use of the computer program SEPP[20,21] are shown in Table 1. The detector equipped with a flow-through cell of 25-mm path length allowed us to obtain the CD and the UV information simultaneously as a function of time. Both the first eluted enantiomer of thalidomide ((*S*) configuration, Figure 2a) and the phenolic metabolite (Figure 2b) show negative CD effects at 240 nm. Accordingly, for the second eluted antipodes of the diastereomeric 5′-hydroxythalidomides (Figure 2c/d), a negative differential absorbance ΔA at the same wavelength can be observed. Since the metabolites presently examined are structurally similar to the reference compound thalidomide (strict analogy of the chromophoric environment), one might conclude

Table 1. Separation Characteristics of Thalidomide and Its Hydroxylated Metabolites, Calculated Using the Computer Program SEPP[a]

compound	v_1 (mL)	v_2 (mL)	k_1	k_2	α	R
TD	13.4	29.3	2.32	6.28	2.71	10.40
5-OH-TD	10.0	13.8	1.48	2.43	1.64	4.47
cis-5'-OH-TD	8.2	15.0	1.03	2.73	2.65	5.72
trans-5'-OH-TD	8.5	15.1	1.10	2.74	2.49	5.98

[a] Experimental conditions: Chiralpak AD 250 × 4.6 mm with precolumn; MeOH.

that the (*S*) absolute configuration can be assigned to the enantiomers showing a negative CD band at 240 nm. This empirical approach implies that the additional stereocenter at C-5′ does not largely influence the CD behavior of the molecule, for example, by a conformational change of the preferential conformation; for this reason, spectra−structure relationships with reference to a molecule of known structure had to be verified by quantum chemical CD calculation as described in the following section.

In addition to the detection at one single wavelength, the CD instrument was used to monitor the approximate CD and UV spectra between 220 and 420 nm of the four racemic compounds separated by LC (Figure 3). These measurements were performed during a temporary stop of the flow; the pure enantiomers stemming from the racemic mixtures were trapped in the chromatographic cell. The CD spectra obtained for thalidomide and its three hydroxylated metabolites were almost identical, consisting of a single band maximum located at 255 nm.

In addition, for better comparison with the calculated CD spectra, the experimental spectra were extended to the more decisive lower wavelengths below 220 nm. This was achieved by stand-alone CD measurements of authentic (*R*)- and (*S*)-thalidomide reference substances (Figure 4a) and stopped-flow measurements of racemates of *cis*-5′-hydroxythalidomide and 5-hydroxy-

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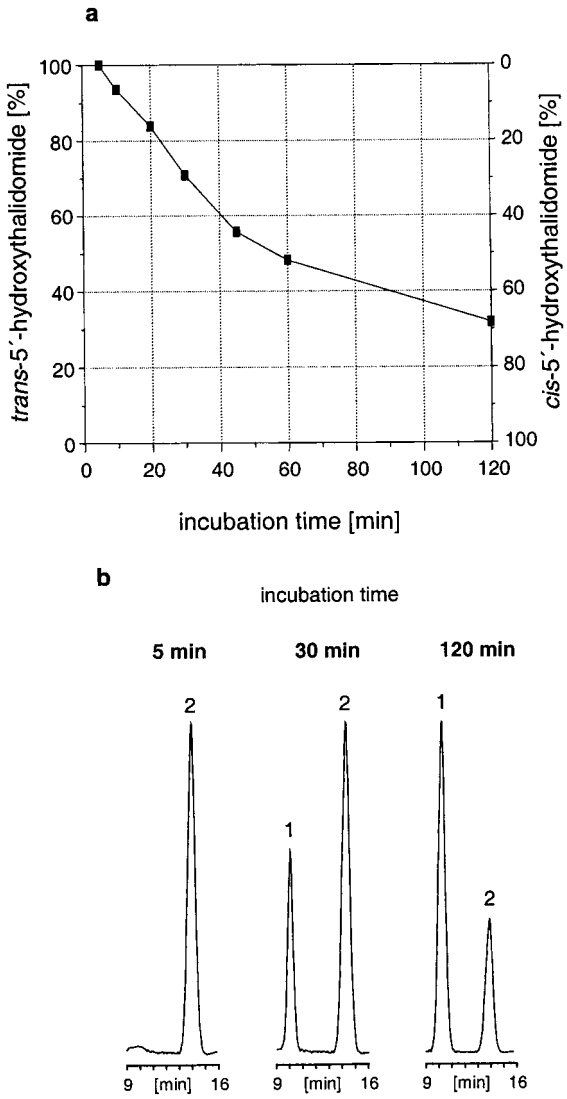

Figure 7. Quantitative relationship of diastereomeric 5′-hydroxythalidomides formed during the in vitro biotransformation of (*R*)-thalidomide with rat liver microsomes, calculated by integration of their peak areas (a). Chromatograms of diastereomeric 5′-hydroxythalidomides, extracted from incubations of (*R*)-thalidomide with rat liver microsomes in the time course of 5, 30, and 120 min (b). Column: LiChrospher 100 RP-18, 125 × 4 mm with a 4 × 4 mm precolumn. Eluent: 1% acetic acid/acetonitrile (92:8, v/v). Flow rate: 1.2 mL/min. Peak assignments: single enantiomers of *cis*-5′-OH-TD (1) and *trans*-5′-OH-TD (2).

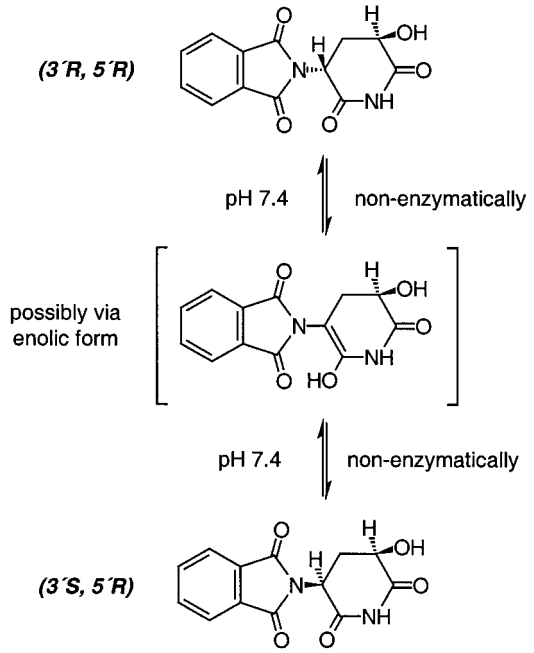

Figure 8. Postulated scheme for the pH-dependent epimerization of (*3′R,5′R*)-5′-hydroxythalidomide via an enolic form as intermediate. Epimerization could also proceed via deprotonation at the chiral center C-3′ and formation of an enolate anion.

thalidomide. In Figure 4b, the resulting spectra featuring a negative CD band at 255 nm are compared.

Quantum Chemical Calculation of CD Spectra. A central question decisive for the correct interpretation of the CD spectra of the 5- and the 5′-hydroxy derivatives (Figure 3) by comparison with the spectra of thalidomide itself was whether the configuration at C-3′ or that at C-5′ is responsible for the sign of the CD curves of the 5′-hydroxy derivatives. To learn whether the curve shape is still determined by the stereocenter at C-3′ or whether it is influenced by the additionally introduced hydroxy group at C-5′, we calculated the CD spectra for all of the four possible (*3′S*) compounds by quantum chemical methods, which had proved to be a particularly valuable tool in earlier studies.[39−41] For a first method validation, we started by investigating the configurationally well-known parent compound thalidomide itself using our established tool for the stereochemical study of novel molecules—the MD approach.[39,40] The CD calculations were based on a MD simulation using the Tripos force field,[31] arbitrarily starting with the (*3′S*) enantiomer. The simulation was performed for a total time period of 500 ps, recording the structure every 0.5 ps for further calculations.

For the 1000 structures thus collected, single CD spectra were calculated and then averaged arithmetically to give the theoretical overall spectrum. To take into account systematic shifts of the calculated CD spectra, a "UV correction" was carried out for each calculated spectrum as introduced earlier.[41] Except for the lacking band at 255 nm in the calculated overall CD spectrum for (*3′S*)-thalidomide, it agrees well with the experimental one of the (*3′S*)-configured reference compound (Figure 5a), especially in the diagnostically decisive short-wavelength part of the CD spectrum.

Comparing the resulting CD spectra of all the (*3′S*) enantiomers thus calculated (Figure 5b), one cannot state any significant changes in their curve shapes, except for small differences in the intensities between the curves and for slight shifts of some of the bands. In view of the divergence of the calculated and the experimental CD spectra in the long-wavelength region, we additionally performed higher-level calculations, viz conformational analyses based on density functional theory (DFT) methods. For thalidomide itself, one minimum structure ("AX", with the nitrogen substituent axial) located 2.30 kcal/mol higher than the global minimum ("EQ", with the nitrogen substituent equatorial) was found.

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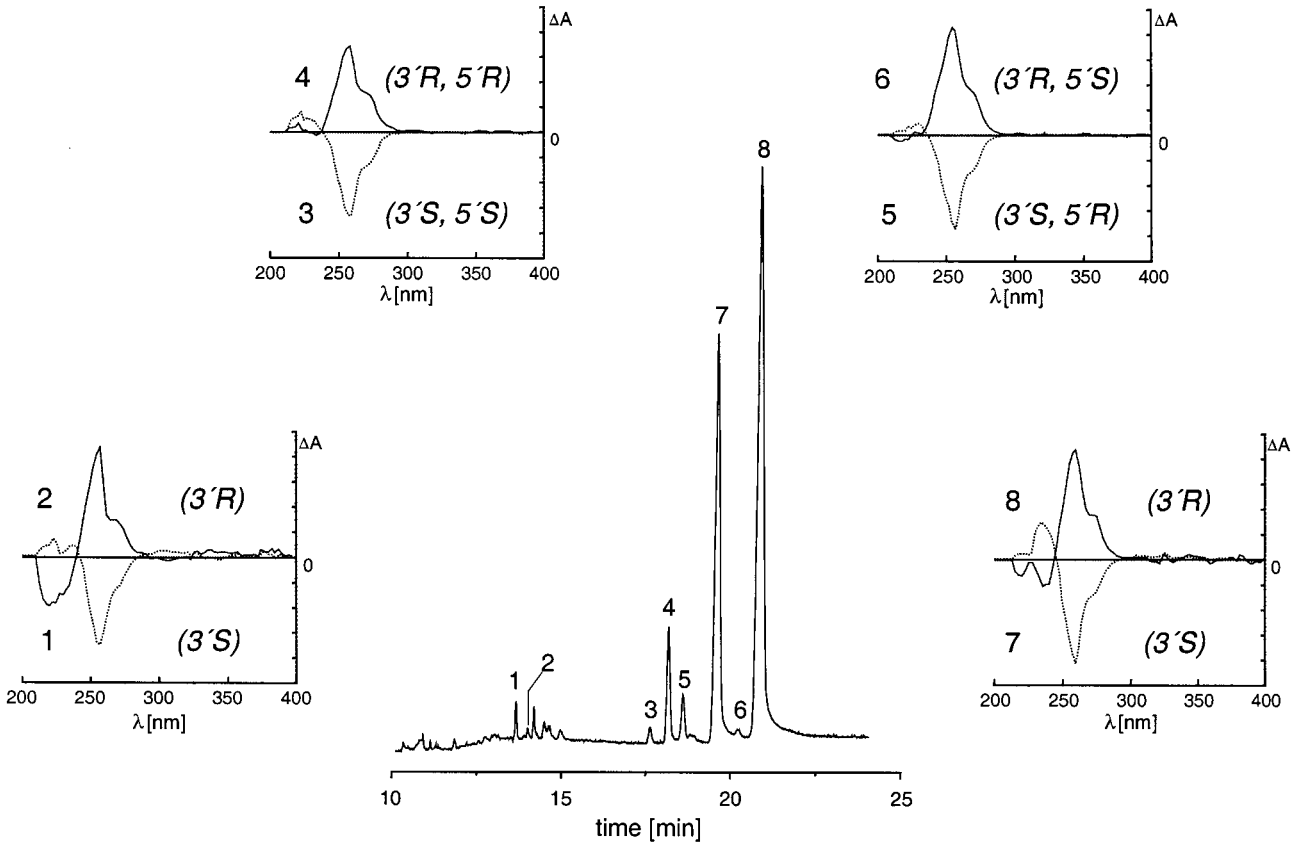

Figure 9. Electropherogram of thalidomide and its hydroxylated metabolites, extracted from incubations of the racemic substrate, and correlation with their CD spectra obtained during LC separation of racemates (see Figure 3). Capillary: PAA-coated, 50-cm i.d., 50-cm EL, acetate buffer pH 4.5, 12 mg/mL β-cyclodextrin and SBE(4)-β-cyclodextrin as chiral selectors, −30 kV (reversed polarity). Peak assignments: racemates of 5-OH-TD (1, 2), *trans*-5′-OH-TD (3, 4), *cis*-5′-OH-TD (5, 6), and TD (7, 8).

For each of the two conformers detected, individual CD spectra were calculated separately. These single CD spectra were Boltzmann-weighted according to the enthalpies of formation of the corresponding conformers and added up to yield the theoretical overall CD spectrum of (*3′S*)-thalidomide. This spectrum was subsequently UV corrected as performed during the MD investigation. Comparing the experimental CD spectrum of (*3′S*)-thalidomide with the DFT−Boltzmann calculated one (Figure 5a), one can see a still better agreement than using the MD approach above: this time, in addition to the mentioned short-wavelength bands also the next minimum at 255 nm is reproduced.

For the *cis*-(*3′S,5′R*)-5′-hydroxythalidomide and the (*S*)-5-hydroxythalidomide in the same way, we calculated the two CD spectra shown in Figure 5d, in comparison to that of (*S*)-thalidomide. In the case of (*S*)-5-hydroxythalidomide, we found 4 AX and 4 EQ minimum geometries below an energetic cutoff of 3 kcal/mol. The EQ minimums are ∼2.30 kcal/mol more stable than the AX minimums. *cis*-(*3′S,5′R*)-5′-Hydroxythalidomide forms only one EQ minimum structure below the cutoff. All the three EQ minimum structures are very similar, so that the resulting overall CD spectra of the DFT−Boltzmann-based approach do not differ largely from each other. The difference of the experimental CD spectra of the hydroxylated compounds compared with that of thalidomide itself (as shown in Figure 4b) may be due to additional solvent effects (resulting from the OH group not present in the parent molecule) not taken into account in our calculations.

All the findings discussed above evidence that the introduction of a hydroxy group at any of the three positions—C-5, *cis*-C-5′, or *trans*-C-5′—*does not substantially affect* the main trend of the CD curves. Consequently, one can deduce that all of the metabolites whose CD spectra feature the same sign around 255 nm as that of thalidomide itself (see Figure 3) must have the same absolute configuration at C-3′.

Analysis of the Incubation Extracts by Circular Dichroism Spectroscopy On Line with Liquid Chromatography. Former investigations of the in vitro biotransformation of (*R*)-thalidomide by rat liver microsomes using chiral capillary electrophoresis demonstrated that the hydroxylated metabolites were formed enantioselectively.[15] Based on these results, on-line CD spectroscopy during chromatographic resolution on an achiral stationary phase (LiChrospher 100 RP-18) was applied to incubation extracts.

(*R*)-Thalidomide as well as its 5- and *trans*-5′-hydroxylated metabolites show positive differential absorbances ΔA at 250 nm (Figure 6a). From the sign of these CD bands and the knowledge gained by the above CD calculations it is possible to establish the (*R*) configuration at the chiral center C-3′. As the relative configurations of diastereomeric 5′-hydroxythalidomide pairs were previously determined either as (*3′R,5′R*) or (*3′S,5′S*) for the trans and (*3′R, 5′S*) or (*3′S, 5′R*) for the cis isomer,[15] the *trans*-5′-hydroxythalidomide metabolite was assigned to have a (*3′R,5′R*) absolute stereostructure. The negative CD of *cis*-5′-hydroxythalidomide consequently corresponds to the (*3′S, 5′R*) configuration.

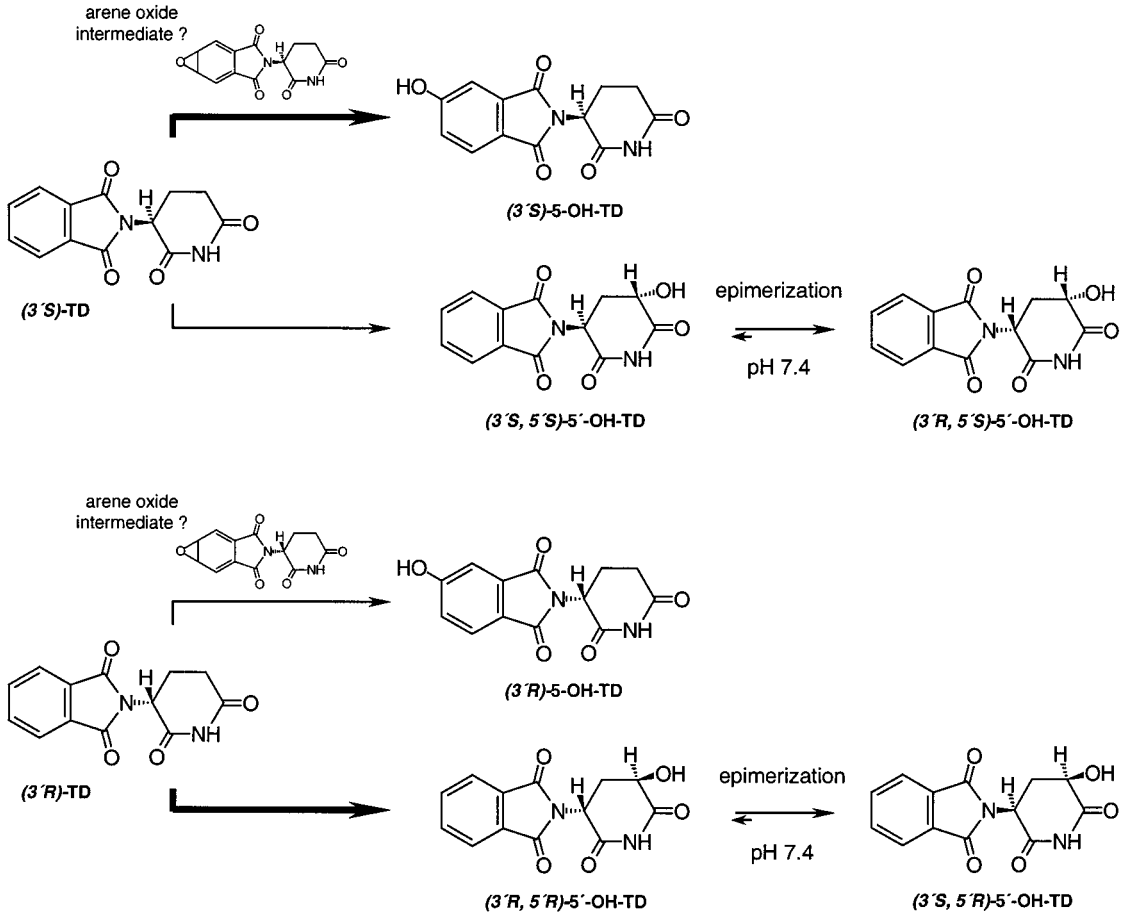

Figure 10. Structures and absolute configurations of the hydroxylated metabolites of thalidomide enantiomers found in incubations with rat liver microsomes. The enantiomers of *cis*-5′-hydroxythalidomide (secondary products) are formed by nonenzymatic epimerization.

The question of whether this isomer is formed enzymatically or by epimerization of *trans*-5′-hydroxythalidomide has to be dicussed later.

A distinguishable pattern of biotransformation products was previously found for (*S*)-thalidomide.[15] The main metabolite of this enantiomer, namely, 5-hydroxythalidomide, and *trans*-5′-hydroxythalidomide were detected in enantiomeric ratios of 95:5 and 92:8, respectively. The analysis of an incubation extract of (*S*)-thalidomide by chiroptical detection during LC is shown in Figure 6b. The negative CDs of the enantiomeric substrate and the 5-hydroxy metabolite formed in excess correspond to the (*S*) absolute configuration. The sensitivity of the CD instrument was insufficient for the detection of the minor metabolite *trans*-5′-hydroxythalidomide. Additionally, the use of an optically inactive sorbent did not allow to monitor the CD of the enantiomers of the cis isomer, which are present in nearly racemic ratios[15] in this incubation extract.

Epimerization of Diastereomeric 5'-Hydroxythalidomides. The diastereomers of 5′-hydroxythalidomide formed in the metabolism of (*R*)-thalidomide were detected in different amounts according to the period of incubation. Reincubations of the racemic standards proved a mutual transformation of both diastereomers at pH 7.4, the equilibrium being shifted toward the cis form.[15] The question of whether this thermodynamically more stable isomer is a primary product during the enzyme-catalyzed biotransformation could not be answered reliably yet.

Therefore, the time-dependent metabolic formation was investigated by incubating (*R*)-thalidomide for various periods (5−120 min). The amounts of the stereosiomers of 5′-hydroxythalidomide separated on an achiral RP-18 column were calculated by integration of their peak areas (Figure 7a), assuming that their absorption coefficients are very similar. The corresponding chromatograms of three different incubation extracts are shown in Figure 7b. These results demonstrate the formation of *trans*-5′-hydroxythalidomide as a primary metabolite (5 min). As an induction period in this microsomal preparation system should be very unlikely, the cis isomer must be a product of the pH-dependent epimerization exclusively. The known absolute configuration of these enantiomeric thalidomide derivatives assigned by means of CD spectroscopy enabled us to determine that an inversion occurs at the chiral center C-3′ of the molecule: (*3′R,5′R*)-*trans*-5′-hydroxythalidomide epimerizes to the thermodynamically more stable (*3′S, 5′R*)-*cis* isomer (Figure 8). Previous hypotheses[15] were disproved by the present investigations.

Stereochemical Aspects of the in Vitro Biotransformation of Thalidomide. The capillary electrophoresis method using a dual cyclodextrin system[15] provided the first example for the enantioselective resolution of thalidomide and its hydroxylated metabolites in a single run. The electropherogram of this chiral separation is illustrated in Figure 9. The analysis of reference compounds as well as incubation extracts of thalidomide enantiomers by circular dichroism spectroscopy presents the opportunity

to assign the CD spectra to the enantiomers of each metabolite separated by CE. The known absolute configuration of these chiral molecules allowed us to follow the stereo- and enantioselective effects of the thalidomide metabolism in detail. The current results are in agreement with previous observations and demonstrate that (*R*)- and (*S*)-thalidomide were predominantly hydroxylated at different moieties of the molecule. The phenolic 5-hydroxy metabolite of (*S*)-thalidomide was unambiguously identified to have the (*S*) absolute configuration (peak 1). The evidence for the formation of an intermediate arene oxide has been presented previously. The optical antipode (*R*)-5-hydroxythalidomide was formed from *(R)*-thalidomide but in substantially lower amounts. Hydroxylation in the glutarimide moiety leading to diastereomeric products was proved to be the major metabolic pathway of this enantiomer. The primary metabolite *trans*-5′-hydroxythalidomide was assigned to have (*3′R,5′R*) absolute configuration (peak 4). This stereoisomer epimerizes nonenzymatically by inversion at the chiral center C-3′. Both incubation time and pH of the incubation medium have a determining influence on this epimerization. The *(3′S,5′R*) absolute stereochemistry of this secondary product (peak 5) follows from the known relative configuration and the negative CD band at 250 nm.

The major and minor metabolites of each thalidomide enantiomer as well as their secondary products are shown in Figure 10. The present investigations confirm the former assumption[15] that no inversion occurs at the asymmetric center C-3′ of the substrate during the cytochrome P-450-catalyzed biotransformation.

CONCLUSIONS

Former investigations of the in vitro biotransformation of thalidomide using chiral capillary electrophoresis in combination with X-ray crystallography for the stereochemical characterization of metabolites were extended to the application of circular dichroism spectroscopy. This CD-based detection system enables the direct analysis of chiral molecules during HPLC separation on a nonracemic sorbent. The sensitivity of the CD instrument was found to be sufficient for the direct determination of even low concentrations of metabolites in biological matrixes, e.g., in incubation extracts of rat liver microsomes. The CD data of the nonracemic metabolites of (*R*)- and (*S*)-thalidomide were obtained during chromatographic resolution on the achiral sorbent RP-18 silica and compared to the calculated CD spectra. These results together with previous observations[15] allowed an unequivocal assignment of the absolute configurations of the hydroxylated thalidomide metabolites as well as a reliable determination of the enantioselective effects in metabolism.

This study represents an important step toward a wider use of chiroptical detection during liquid chromatography and points out some features that are unique to a detection system based on circular dichroism.

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