

STRUCTURE AND STEREOCHEMISTRY OF THE ACTIVE METABOLITE OF CLOPIDOGREL

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ABSTRACT:

Clopidogrel (SR25990C, PLAVIX) is a potent antiplatelet drug, which has been recently launched and is indicated for the prevention of vascular thrombotic events in patients at risk. Clopidogrel is inactive *in vitro*, and a hepatic biotransformation is necessary to express the full antiaggregating activity of the drug. Moreover, 2-oxo-clopidogrel has been previously suggested to be the essential key intermediate metabolite from which the active metabolite is formed. In the present paper, we give the evidence of the occurrence of an *in vitro* active metabolite after incubation of 2-oxo-clopidogrel with human liver microsomes. This metabolite was purified by liquid chromatography, and its structure was studied by a combination of mass spectrometry (MS) and NMR experiments.

MS results suggested that the active metabolite belongs to a family of eight stereoisomers with the following primary chemical structure: 2-[1-[1-(2-chlorophenyl)-2-methoxy-2-oxoethyl]-4-sulfanyl-3-piperidinylidene]acetic acid. Chiral supercritical fluid chromatography resolved these isomers. However, only one of the eight metabolites retained the biological activity, thus underlining the critical importance of associated absolute configuration. Because of its highly labile character, probably due to a very reactive thiol function, structural elucidation of the active metabolite was performed on the stabilized acrylonitrile derivative. Conjunction of all our results suggested that the active metabolite is of S configuration at C 7 and Z configuration at C 3-C 16 double bond.

Clopidogrel (SR25990C, PLAVIX) is a potent antiaggregant and antithrombotic drug, as demonstrated in several experimental models of thrombosis (Herbert et al., 1993a). The drug was launched on the market following a successful clinical evaluation (Feliste et al., 1987) and demonstration of superior efficacy versus aspirin in preventing thrombotic events (myocardial infarction, stroke, and vascular death) in high risk patients (CAPRIE Steering Committee, 1996).

Clopidogrel inhibits platelet aggregation *ex vivo* induced by ADP, low concentrations of thrombin, or by collagen (CAPRIE Steering Committee, 1996). The specific pharmacological target of clopidogrel is the ADP-induced platelet activation process (Herbert et al., 1993b), and it has been described as a specific and irreversible inhibitor of 2-methyl-S-ADP binding to its platelet receptors, the purinergic P2Y₁₂ receptor (Savi et al., 1994a; Herbert et al., 1999; Savi et al., 2001). Clopidogrel is not active *in vitro*, and a biotransformation by the liver is necessary to allow the expression of its antiaggregating activity (Savi et al., 1992). Therefore, clopidogrel can be considered as a precursor of an active metabolite. Moreover, no antiaggregating activity was found in platelet poor plasma of SR25990C-treated animals or humans, indicating a high reactivity and instability of the active metabolite.

Clopidogrel has an absolute S configuration at carbon 7 (see chemical structure in Fig. 1). The corresponding R enantiomer is totally devoid of antiaggregating activity (Savi et al., 1994b), thus indicating the importance of the configuration of this asymmetric carbon for the biological activity. In previous experiments, incubation of clopidogrel with rat

hepatic microsomes was found to generate 2-oxo-clopidogrel, through a CYP450-dependent pathway of metabolism (Savi et al., 1992, 1994b). Similar results were obtained using human liver microsomes (Savi et al., 2000). Despite being not active *in vitro*, 2-oxo-clopidogrel can demonstrate an antiaggregating activity *ex vivo*, thus indicating that the formation of the active metabolite of clopidogrel occurred downstream to the formation of 2-oxo-clopidogrel (Savi et al., 2000). The structure of active metabolite of another thienopyridine, CS-747, was reported (Sugidachi et al., 2000, 2001). In another report, these authors indicated the precise absolute configuration to express the biological activity, since only one among the four optical isomers showed activity in inhibiting platelet aggregation (Kazui et al., 2001). However, to our knowledge, no structural and stereochemical characterization data were published in detail concerning this active metabolite.

The objective of this work was to identify the chemical structure of the active metabolite of clopidogrel. For this purpose, metabolites generated after incubation of human liver microsomes with 2-oxo-clopidogrel and its corresponding inactive R enantiomer were isolated and purified using a two-step liquid chromatographic procedure. The biological activity of the metabolites was evaluated through the inhibition of binding of radiolabeled 2-methyl-S-ADP to rat platelets. Subsequently, the structure and stereochemistry of the metabolites were studied by a combination of mass spectrometry (MS¹), NMR, and chiral supercritical fluid chromatography (chiral SFC).

¹ Abbreviations used are: MS, mass spectrometry; SFC, supercritical fluid chromatography; PRP, platelet-rich plasma; HPLC, high-performance liquid chromatography; ESI, positive electrospray ionization; amu, atomic mass unit(s); MS/MS, tandem mass spectrometry; EI, electron impact; CI, chemical ionization; DQF, double quantum-filtered correlation spectroscopy; ROESY, rotating frame

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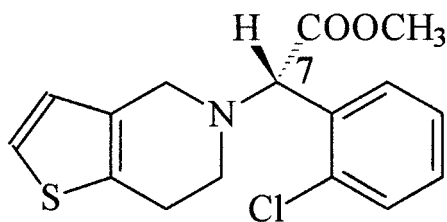


FIG. 1. Chemical structure of clopidogrel.

Materials and Methods

Chemicals. Clopidogrel (SR25990C, 2-(2-chlorophenyl)-2-(2,4,5,6,7,7a-hexahydrothieno [3,2c]pyridine-5yl)-acetic acid methylester hydrogen sulfate, 7S) and its corresponding inactive levogyre enantiomer (SR25989C, 7R) were made available from Sanofi-Synthelabo Recherche (Toulouse, France). 2-Oxo-clopidogrel [(7S) SR121683] and 2-oxo-SR25989C [(7R) SR121682] were prepared by semipreparative chiral HPLC from the racemic (7R,S) SR25552, which was synthesized by Sanofi-Synthelabo Recherche. ^{33}P -2-Methyl-S-ADP (600 Ci/mmol) was from PerkinElmer Life Sciences (Le Blanc Mesnil, France). Glutathione, reduced form, and β -nicotinamide adenosine diphosphate, reduced form (NADPH), were from Sigma-Aldrich (L'Île d'Abeau, France). All other chemicals were of analytical or HPLC grade.

Purification of Clopidogrel Metabolites from Incubation of 2-Oxo-precursors with Human Liver Microsomes. Human liver microsomes from BD Gentest (Woburn, MA) were adjusted to 0.75 mg of protein/ml in 100 mM potassium phosphate buffer, pH 7.4, containing 100 mM KF and 10 mM glutathione. SR121682 and SR121683 were added at a final concentration of 0.1 mM, and the reaction was initiated with 1 mM NADPH. Incubation was carried out at 37°C under continuous stirring (100 rpm) and light protection using a reciprocal incubator. After 60 min, the incubation medium was cooled at 4°C and centrifuged at 10,000g for 10 min.

A first purification step for isolation of metabolites was performed by semipreparative liquid chromatography on a system from Pharmacia AB (Uppsala, Sweden). The incubation supernatants were loaded on a HR16/5 precolumn (50 × 16 mm) from Pharmacia AB, filled with a 10- μm C₁₈ support from Millipore Corporation (Epernon, France). The precolumn was rinsed and equilibrated with a 10 mM ammonium acetate buffer (pH 6.5). The precolumn was then coupled to a 10- μm Kromasil C₁₈ column from Akzo Nobel (Bohus, Sweden). Elution at 2.0 ml/min was performed using an acetonitrile/10 mM ammonium acetate gradient (10 to 90%) and monitored with a dual UV detection (214 nm/254 nm). Eluted fractions were collected and concentrated to 300 μl using a SpeedVac vacuum centrifuge evaporator from Savant Instruments (Holbrook, NY).

The eluted fraction "H" that contains a pool of metabolites was controlled by analytical HPLC using a Superspher 60RP8-E column (125 × 4 mm) from Merck-Clevent (Nogent/Marne, France). Isocratic elution was performed at 0.8 ml/min with 60% methanol in aqueous 0.2% acetic acid/0.1% diethylamine (pH 5.5) and monitored with UV detection at 234 nm.

A second purification step using semipreparative HPLC was performed to separate individual metabolites from the fraction "H", using an Ultrabase UB225 column (250 × 4.6 mm) from SFCC (Neuilly-Plaisance, France). Elution was performed at 1 ml/min using an acetonitrile/10 mM ammonium acetate (pH 6.5) gradient (10 to 24%) with UV detection at 234 nm. The collected fractions were concentrated on the SpeedVac evaporator.

Concentrations of native "H" metabolites were estimated by analytical HPLC, using ticlopidine as an external standard for quantitative calibration. Ticlopidine was chosen because it can be eluted by the same analytical HPLC method as the "H" metabolites, whereas this is not possible with the more hydrophobic clopidogrel. In addition, ticlopidine has the same thiophene and 2-chlorophenyl chromophore groups than clopidogrel. This quantification approach was validated using incubation of radiolabeled ^{14}C -clopidogrel (35.5 $\mu\text{Ci}/\text{mg}$, Isotopic Chemistry Department, Sanofi-Synthelabo Research, Alnwick, UK) to correlate the concentrations measured by UV and specific radioactivity, using the analytical control HPLC method described above.

nuclear Overhauser enhancement spectroscopy; HSQC, heteronuclear single quantum correlation; LC, liquid chromatography.

In Vitro Activity of Clopidogrel Metabolites on the Binding of ^{33}P -2MeS-ADP Human Platelets. Venous blood was collected on citrated tubes from human healthy volunteers. PRP was obtained by centrifugation (120g, 10 min), and PRP samples (2 ml) were incubated for 1 h at 20°C with the purified metabolites. Experiments on the specific binding of ^{33}P -2MeS-ADP to human platelets were performed using a filtration technique to separate the free from bound ^{33}P -2MeS-ADP as previously described (Savi et al., 1994). The preincubated PRPs were centrifuged (600g, 10 min.), then the supernatants were discarded, and the pellets were resuspended in binding buffer (145 mM NaCl, 5 mM KCl, 0.1 mM MgCl₂, 5.5 mM glucose, 15 mM HEPES, 5 mM EDTA). Incubations of the 2-oxo metabolic precursors (SR25552, SR121683, and SR121682) were carried out in 0.2 ml of binding buffer, which contained washed human platelets (0.1×10^9 platelets/ml) and ^{33}P -2MeS-ADP (0.5 nM). Triplicate incubations were carried out at 37°C for 15 min and were terminated by the addition of a 3-ml ice-cold assay buffer followed by rapid vacuum filtration over glass-fiber Filtermats 11734 from Skatron Instruments (Sterling, VA). Filters were then washed twice with 5-ml ice-cold incubation buffer, dried, and the radioactivity was measured by scintillation counting. Nonspecific binding was defined as the total binding measured in the presence of excess unlabeled ADP (1 mM), and specific binding was defined as the difference between total binding and nonspecific binding. The percent inhibition was expressed as %I = (total binding – total binding with antagonist)/specific binding × 100.

Derivatization of Clopidogrel Metabolites with Acrylonitrile. The fraction H containing the metabolites from microsomal incubation with 2-oxo-precursors (SR25552, SR121683, or SR121682) was diluted in an excess of acrylonitrile. After overnight agitation at room temperature, the solutions were evaporated to dryness with a SpeedVac system. The derivatized metabolites were then purified either by semipreparative HPLC as described for native metabolites or by analytical HPLC system using a 5- μm Kromasil C₁₈ column (100 × 4.6 mm) and an acetonitrile/0.1% trifluoroacetic acid gradient (18 to 25%). Concentrations of derivatized metabolites were estimated by analytical HPLC, as described above for native metabolites.

Mass Spectrometry LC/MS and LC/MS/MS of native metabolites. Fraction H from microsomal incubation with the racemic 2-oxo-precursor SR25552 was injected on a Lichocart 60RP8E column (125 × 4 mm) from Merck-Clevent using a HP1100 liquid chromatograph from Agilent Technologies (Waldbronn, Germany). Isocratic elution was performed with a mixture of methanol/water/acetic acid/diethylamine (40:60:0.2:0.1 v/v/v/v) at 0.7 ml/min, and UV signal was followed at 254 nm. MS data were acquired on a Finnigan LCQ instrument from Thermoquest (San Jose, CA) in positive electrospray ionization (ESI⁺) mode. The spray potential was set at 5.6 kV and capillary temperature at 230°C. Mass range was scanned between 100 and 900 amu. In MS/MS mode (23% of collision energy), the two parent ions obtained at m/z 356.5 and 358.5 (with 1.4 amu peak width) correspond, respectively, to the quasi-molecular ions of the metabolites containing either ^{35}Cl or ^{37}Cl isotope.

EI and CI mass spectrometry of methyl-derivatized metabolites. Fraction H from SR25552 was derivatized using ethereal diazomethane reaction. MS analyses using electron impact (EI) and chemical ionization (CI) were done on a Finnigan TSQ 700 mass spectrometer from Thermoquest. EI was performed at 70 eV, and scans were taken over the mass range m/z 40 to 500, whereas CI was conducted using ammonia as reactant gas, and scans were taken over the mass range of m/z 80 to 900. Both direct introductions were done using a probe with a current gradient from 50 to 800 mA in 2 min.

LC/MS of acrylonitrile-derivatized metabolites. Fraction H from SR25552 was derivatized with acrylonitrile and analyzed on the TSQ 700 mass spectrometer using the same conditions as for the native metabolic fraction H.

Chiral Supercritical Fluid Chromatography. The acrylonitrile-derivatized H metabolites from microsomal incubation with either 2-oxo-precursor SR121683 or SR121682 were analyzed by SFC using a chromatograph from Berger Instruments Inc. (Newark, DE). The system comprises a FCM-1200 fluid control module for pumping carbon dioxide and polar modifier, a TCM-2020 column thermal control module, an ALS-3150 autosampler, a DAD-4100 diode-array UV detector, an electronic back-pressure regulator, and a Chem-Station software (Agilent Technologies) for instrument control and data acquisition/processing. Separation was achieved using a Chiralpak-AD column (250 × 4.6 mm) from Daicel Chemicals (Tokyo, Japan) and a carbon dioxide/isopropanol containing 0.4% triethylamine and 0.4% trifluoroacetic acid

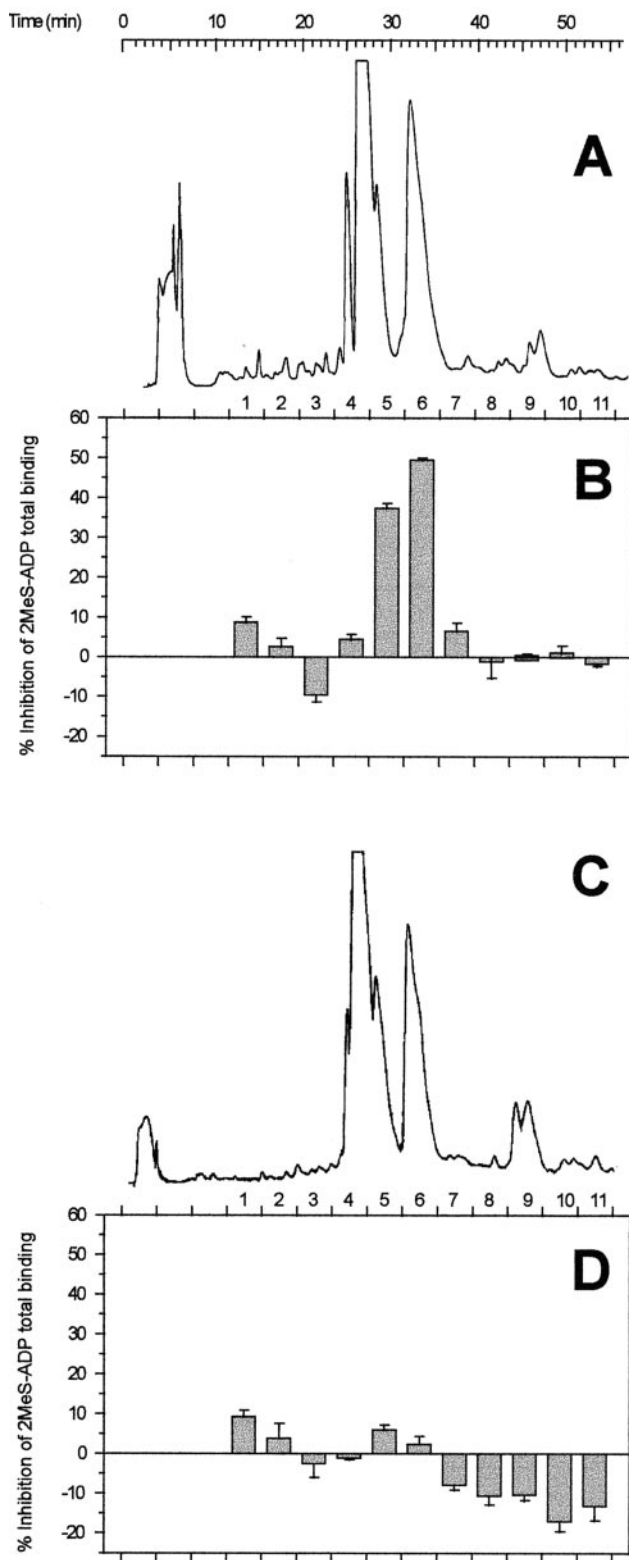


Fig. 2. Semipreparative HPLC purification chromatograms and biological activity of the collected fractions from incubations of (7S) SR121683 (A and B) and (7R) SR121682 (C and D) with human microsomes.

(90:10 v/v) mixture as fluid eluent. The operating conditions were 3 ml/min flow rate, 200 bar outlet pressure, and 5°C column temperature. The sample was dissolved in an isopropanol/methylene chloride (v/v) mixture and 10 μ l were injected. UV detection was carried out at 220 nm.

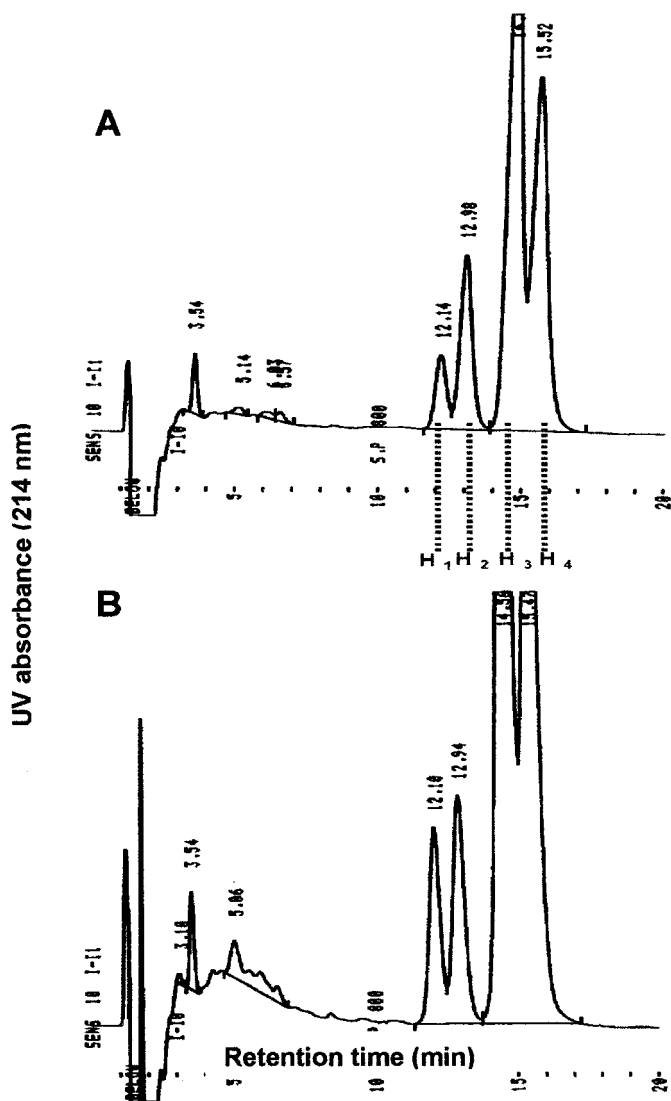


Fig. 3. Analytical HPLC control chromatograms of metabolites pool H from incubation of (7S) SR121683 (A) and (7R) SR121682 (B) with human microsomes.

Nuclear Magnetic Resonance. ^1H (500.13 MHz) and ^{13}C (125.77 MHz) NMR spectra of acrylonitrile-derivatized H metabolites from microsomal incubation with racemic 2-oxo-precursor SR25552 were recorded on an Avance DRX500 spectrometer from Bruker (Karlsruhe, Germany). The probe was a $^1\text{H}/^{13}\text{C}$ 5 mm, 3 axis gradients (x,y,z), optimized for inverse detection. Spectra were recorded in CDCl_3 solvent in 5-mm tubes without spinning at a temperature of 300K. Sample concentration was less than 1 mg in 0.5 ml. The residual protonated resonance of the solvent (CDCl_3) was used as an internal chemical shift standard, which was related to tetramethylsilane with chemical shifts of 7.25 and 77 ppm, respectively, for ^1H and ^{13}C . The pulse programs of all 2D experiments (gradient-selected COSY, gradient-selected DQF-COSY, ROESY and gradient selected $^1\text{H}/^{13}\text{C}$ HSQC) were taken from the Bruker standard software library. Processing of the raw data were performed using Bruker XWinNmr software running on a Silicon Graphics Indy workstation. The pulse conditions were 90° pulse, 6.8 μ s (attenuation 0db) for ^1H and 5 μ s (attenuation -2db) for ^{13}C . Gradient pulses used in this study were all shaped to a sine envelope with 1 ms duration (COSY, DQF-COSY, and $^1\text{H}/^{13}\text{C}$ HSQC). Spectral width was 5530.97 Hz for proton and 34013.6 Hz for carbon.

The main acquisition and processing parameters were as follows: ^1H -1D spectrum, 1024 scans, time domain size = 64K; ^1H -2D gradient selected COSY, 16 scans, time domain size = 2K in t2, 512 experiments in t1, gradient amplitude 10:10 G.cm $^{-1}$, zero-filling up to 2K in t1, sinus filter in t1 and t2;

TABLE 1

Biological activity of native H1 to H4 metabolite fractions (1 μ M equivalent) on 33 P-2-MeS-ADP binding to platelets

Metabolite	Concentration	Inhibition of 33 P-2-MeS-ADP binding
	μ M	
H1	Not determined	0
H2	Not determined	0
H3	39.7	0
H4	22.2	83

1 H-2D gradient selected DQF-COSY, 64 scans, time domain size = 2K in t2, 512 experiments in t1, gradient amplitude 15:30 G.cm $^{-1}$, zero-filling up to 2K in t1, cosine2 filter in t1 and t2; 1 H-2D ROESY spectrum, 32 scans, time domain size = 2K in t2, 512 experiments in t1, 300 ms mixing time, zero-filling up to 2K in t1, cosine2 filter in both t1 and t2; 13 C-1D decoupled spectrum: 10 000 scans, time domain size = 16K, decoupling 1H using WALTZ16, exponential filter with a time constant of 3Hz before Fourier transformation; 1 H/ 13 C 2D gradient selected HSQC, 64 scans, time domain size = 2K in t2, 128 experiments in t1, gradient amplitude 40:10:40:–10 G.cm $^{-1}$, decoupling 13 C using globally optimized alternating-phase rectangular pulses, delay optimized for an average 1 J_{CH}-coupling constant of 131 Hz, zero-filling up to 2K in t1, cosine2 filter in t1 and t2.

Molecular Modeling. Conformational analysis was performed using the SYBIL software from Tripos Inc. (St. Louis, MO) running on a Silicon Graphics R10000 workstation. The goal of this modelisation was to explore the conformational space of the acrylonitrile-derivatized H metabolites. Molecular dynamics studies were carried out using a method called "high temperature" (600K) during 1 ps. Results were used to find a minimum of energy for each structure. The force field used to perform dynamic experiments and energy minimization were those of Tripos. These calculations allowed finding a privileged conformation for each molecule.

Results

Biotransformation of 2-Oxo-clopidogrel into an Active Metabolite. The active metabolite of clopidogrel (SR25990C) was suspected to be formed downstream to the formation of 2-oxo-clopidogrel following incubation of human liver microsomes with the latter. Parallel incubations were carried out with the 2-oxo-clopidogrel (SR121683) and its opposite inactive R enantiomer (SR121682) as a control. Only SR121683 could demonstrate ex vivo biological activity (Savi et al., 2000), supporting the fact that SR121683 is the 2-oxo-SR25990C and therefore should have an S configuration at carbon 7. The metabolites present in the supernatant of incubates were isolated in a single fraction using semipreparative chromatography. SR121683 and SR121682 generated similar elution patterns (data not shown), indicating an identical metabolic pathway. An inhibitory effect of 33 P-2-methyl-S-ADP binding to platelets was found only in a fraction from the SR121683 incubate, named fraction H.

To determine which peak(s) bore the biological activity, fraction H derived from SR121683 was further separated by semipreparative HPLC (Fig. 2A), and activity was assessed on the eluted fractions by measuring 33 P-2-methyl-S-ADP binding to human platelets (Fig. 2B). Similar purification was performed for fraction H derived from SR121682 (Fig. 2, C and D). As expected, the activity could be detected only in SR121683 derived metabolites (mainly in fractions 5 and 6), metabolites from SR121682 being not active. Fraction H from SR121683 was further resolved by analytical HPLC (Fig. 3A). Four different peaks were observed and named H1, H2, H3, and H4 according to their elution order. The fractions tested for biological activity (Fig. 2A) were checked by the same analytical HPLC method. In the collected fraction 6, only H4 was detected, whereas fraction 4 contained 13% H1 and 87% H3 and fraction 5 contained 51% H2, 28% H3, and 21% H4. Again, a similar four peak analytical HPLC

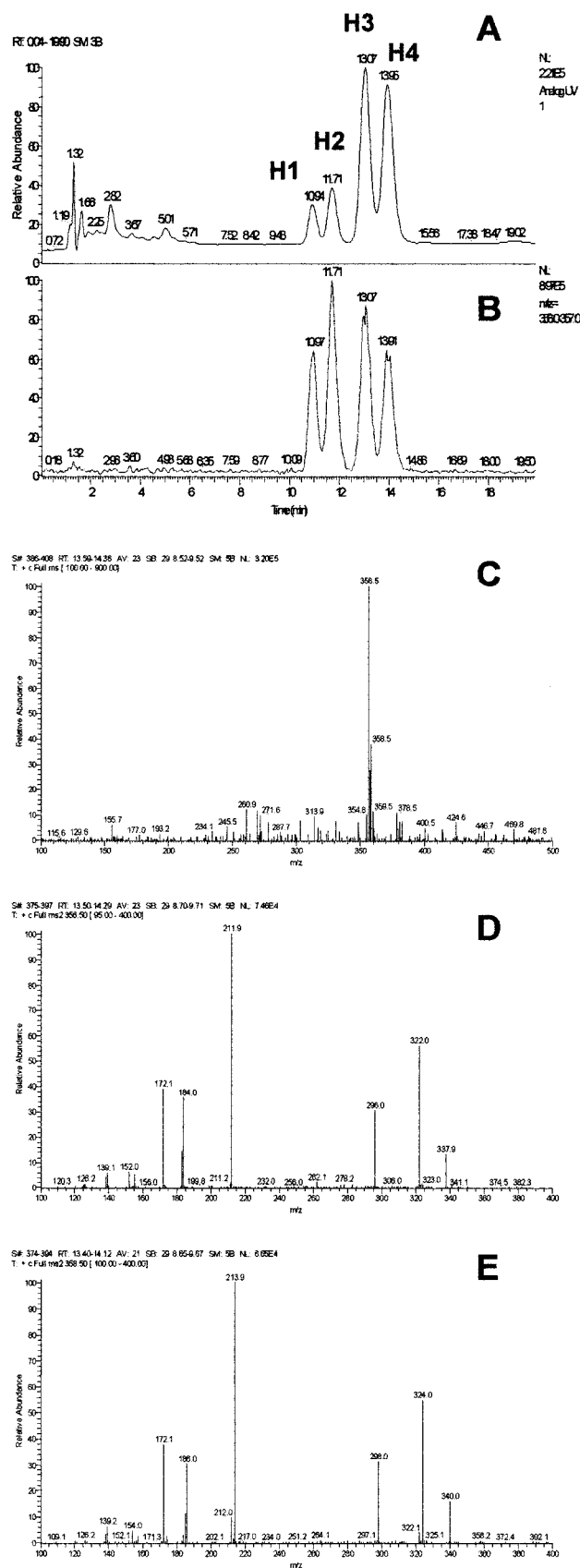


FIG. 4. LC/MS and LC/MS/MS spectra of metabolites pool H, showing UV (A) and MS total current trace for MH $^{+}$ at m/z 356.5 ion (B), ESI $^{+}$ /MS spectrum for peak H4 (C), ESI $^{+}$ /MS/MS spectrum of m/z 356.5 ion (MH $^{+}$ bearing 35 Cl isotope, D) and ESI $^{+}$ /MS/MS spectrum of m/z 358.5 ion (MH $^{+}$ bearing 37 Cl isotope, E).

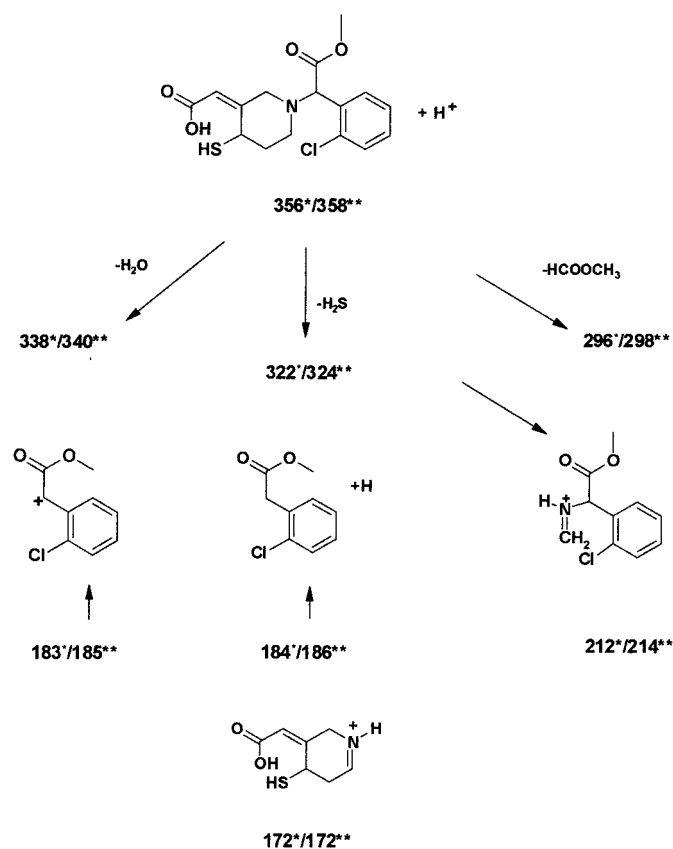


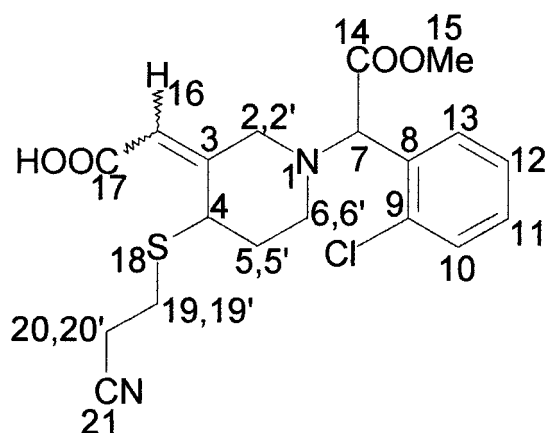
FIG. 5. Primary structure of the active H4 metabolite of clopidogrel and MS fragmentation pathway proposal from ESI⁺/MS/MS data.

Spectra were obtained on MH⁺ bearing ³⁵Cl and ³⁷Cl isotopes. *, selected ion *m/z* 356.5. **, selected ion *m/z* 358.5.

profile with almost the same retention times was obtained with the SR121682-derived metabolic pool (Fig. 3B). The four H1 to H4 peaks from SR121683 incubates were collected and separately checked for their biological activity. Results shown in Table 1 indicate that only the fraction corresponding to the H4 peak contained an active metabolite of clopidogrel.

Structural Elucidation of the Active Metabolite. It was clearly demonstrated that the biological activity was supported by the S stereochemical configuration at carbon 7 of clopidogrel. It was also shown that the two achiral chromatographic profiles of metabolic pool “H” from either oxo-precursor SR12683 or SR121682 microsomal incubates were similar (see Fig. 4). Hence, all the subsequent structural elucidation studies using nonstereoselective MS and NMR spectroscopy were carried out on metabolic pool “H” obtained by human microsomal incubation of the racemic 2-oxo-precursor (7S, 7R) SR25552.

Mass spectrometry. LC/MS experiments were conducted on the metabolic pool “H” from SR25552. Both UV and mass detection (*m/z* 356–357) profiles demonstrated the presence of the four different peaks noted H1 to H4 (Fig. 4, A and B). The detected four peaks in MS had exactly the same characteristics with a molecular ion MH⁺ at *m/z* 356.5, corresponding to 34 amu more than clopidogrel (Fig. 4C). The MS/MS data obtained on quasi-molecular ions MH⁺ 356.5 and 358.5 containing ³⁵Cl or ³⁷Cl isotope, respectively, allowed the detection of fragments bearing a chlorine atom (Fig. 4, D and E). MS/MS data were the same for the four compounds and were found in accordance to the primary structure proposal and the fragmentation scheme shown in Fig. 5.



SCHEME 1. Z or E isomer.

To confirm the presence of a carboxylic acid function, metabolic pool “H” from SR25552 was derivatized with diazomethane. Results obtained in EI and CI ionisation modes confirmed the incorporation of two methyl groups in each of the four structures. The molecular M⁺ ion was observed at *m/z* 383 in the EI mass spectrum and MH⁺ ion at *m/z* 384 in DCI⁺/NH₃ mass spectrum (data not shown). These results were in good accordance with the proposed primary structure for clopidogrel metabolites having a carboxylic acid function and a thiol group.

Nuclear Magnetic Resonance. From the four diastereomer H1 to H4 metabolites generated from incubation of SR25552 with human microsomes and purified by HPLC, only H4 was shown to retain the biological activity. This underlines the importance of a specific and critical absolute configuration for the active metabolite, the exact nature of which remained to be elucidated using NMR. However the active H fraction isolated by semipreparative chromatography or obtained after subsequent semipreparative HPLC purification, appeared to be highly labile since its activity was quickly lost after overnight incubation at room temperature. This could be explained by the presence of the reactive thiol group on the proposed structure (Fig. 5). Therefore, for all subsequent NMR studies, the “H” metabolites were always stabilized by trapping the reactive thiol group with acrylonitrile a thiol specific reagent, which was chosen because it did not introduce a supplementary asymmetric carbon in the chemical structure of the compound. The resulting acrylonitrile derivatives were controlled by LC/MS before use. They always gave the four HPLC separated H1 to H4 analog peaks, all of which exhibited a quasi-molecular ion MH⁺ at *m/z* 409 thus confirming the introduction of an acrylonitrile group on the thiol function.

Assignments of the resonance to individual proton and carbon nuclei of the molecules were performed according to the following numbering NMR nomenclature (Scheme 1).

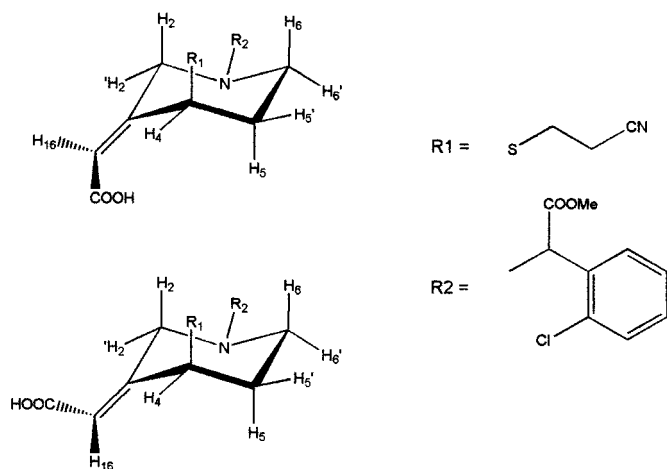
The set of NMR data were fully compatible with the chemical structure proposed above. All assignments were carried out using chemical shift tables, homonuclear ¹H coupling detected by the presence of cross peaks on correlation spectra (COSY, DQF-COSY), heteronuclear ¹H/¹³C coupling detected by the presence of cross peaks on HSQC spectrum, and homonuclear ¹H dipolar coupling obtained from ROESY experiments. Table 2 shows ¹H and ¹³C chemical shift assignments of derivatized H1, H3, and H4 metabolites. Interpretable spectra for the derivatized H2 metabolite were not obtained due to insufficient amount of purified compound.

Conformational analysis and configuration of the ethylenic bond. Molecular modeling calculations were performed taking into account NMR constraints (¹H homonuclear long range couplings and nuclear Overhauser effects). Conformations corresponding to energy minima

TABLE 2
¹H- and ¹³C-NMR chemical shifts of acrylonitrile-derivatized H1, H3, and H4 metabolites

Nuclei Number	Derivatized H1 Metabolite		Derivatized H3 Metabolite		Derivatized H4 Metabolite	
	$\delta^1\text{H}$ (multiplicity, integral)	$\delta^{13}\text{C}$	$\delta^1\text{H}$ (multiplicity, integral)	$\delta^{13}\text{C}$	$\delta^1\text{H}$ (multiplicity, integral)	$\delta^{13}\text{C}$
1 (N)						
2	3.57 (d, 1H)	46.8	3.56 (d, 1H)	53.9	3.66 (d, 1H)	54.3
2'	4.37 (d, 1H)		2.94 (d, 1H)		3.15 (d, 1H)	
3		ND		157.5		157.3
4	3.61 (s, 1H)	48.3	5.17 (d, 1H)	38.3	5.18 (d, 1H)	38.5
5	2.26–2.41 (m, 1H)	31.6	2.22–2.31 (m, 1H)	31.3	2.15–2.25 (m, 1H)	31.2
5'	1.87 (d, 1H)		1.92 (d, 1H)		1.83–1.89 (m, 1H)	
6	2.73–2.84 (m, 2H)	46.1	2.86 (d, 1H)	46.3	2.62–2.67 (m, 2H)	46.1
6'			2.74 (m, 1H)			
7	4.88 (s, 1H)	67.1	4.80 (s, 1H)	67.8	4.80 (s, 1H)	67.8
8		134.7		134.6		134.8
9		133.2		133.6		133.1
10						
11	7.22–7.58 (m, 4H)	127.1	7.24–7.62 (m, 4H)	126	7.25–7.58 (m, 4H)	127.2
12		to		to		to
13		130		130		130.1
14		171.2		171.1		170.9
15	3.71 (s, 3H)	52.2	3.70 (s, 3H)	51.9	3.70 (s, 3H)	52.3
16	5.78 (s, 1H)	118.1	5.70 (s, 1H)	115.6	5.80 (s, 1H)	116
17		ND		169.3		169.2
18 (S)						
19		26.7		26.5		26.5
19'	2.61–2.66 (m, 4H)		2.60–2.73 (m, 4H)		2.68–2.75 (m, 4H)	
20		18.7		18.8		19
20'						
21		ND		118.2		118.4

ND, not determined; s, singlet; d, Doublet; m, multiplet.



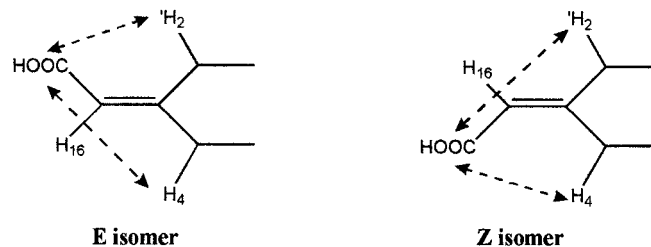
SCHEME 2. Calculated structure for both E and Z double bond configuration.

were sought. In these conformers, the piperidine group adopted a chair conformation with the $\text{SCH}_2\text{CH}_2\text{CN}$ group in axial position. Indeed, it seems that the equatorial position was unfavorable for the $\text{SCH}_2\text{CH}_2\text{CN}$ group (+3 kcal) due to steric constraints induced by the bulky sulfur group.

Scheme 2 shows the calculated structure for both E and Z double bond configuration, together with estimated internuclei distances. For derivatized H3 and H4 metabolites, ROESY experiments exhibit cross peak between protons 2' and 16 but no cross peak between proton 4 and 16. For derivatized H1, ROESY experiments exhibit a cross peak between protons 4 and 16 and no cross peak between protons 2' and 16. It was then deduced that H3 and H4 have the same Z configuration, H1 being of the E configuration. This is confirmed by a close examination of chemical shifts, as highlighted in Table 3. Variations

on chemical shifts of protons 2' and 4 appears reverse in H1 compared with H3 and H4. This can be explained by Scheme 3:

In the case of Z isomer, the observed chemical shift for proton 4 (i.e., 5.18 ppm) was unusually high, indicating that the carbonyl group had an impact on the proton 4, due to its spatial proximity. In the case of



SCHEME 3. E isomer and Z isomer.

E isomer, the carbonyl group has no effect on the proton 4, resulting in usual chemical shift for this proton (3.61 ppm). Conversely, in the case of E isomer, the observed chemical shift for proton 2' was unusually high (4.37 ppm) indicating that the carbonyl group had an effect on this proton. In the case of Z isomer, the carbonyl group could not have an effect on this proton, resulting in a normal chemical shift for proton 2' (3.66 ppm). The same phenomenon is visible on ¹³C chemical shifts of carbons 2 and 4 (Table 2).

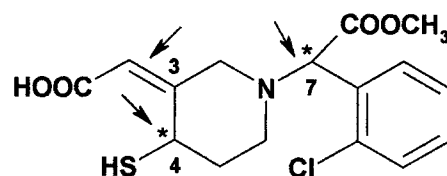
In summary, NMR data sets were in accordance with Scheme 4. Acrylonitrile-derivatized H3 and H4 metabolites corresponded to the structure having the Z stereochemistry on the ethylenic double bond. Chemical shifts of derivatized H3 and H4 metabolites show a similar but slightly different structure. This was presumably due to the two diastereomers arising from the asymmetric carbon 4. H1 metabolite has been shown to possess E stereochemistry at the ethylenic double bond. We have been unable to obtain good spectra from the derivat-

ized H2 metabolite, but we can reasonably postulate that H1 and H2 are both of E configuration, and, like H3 and H4, diastereomers differing from the absolute configuration at carbon 4.

SFC Separation of Stereoisomer Metabolites. MS and NMR data are in accordance with Scheme 5. This chemical structure contains three stereochemical sites: two chiral centers at C 4 and C 7 and one geometric center at C 3 (ethylenic bond). Eight stereochemical isomers can be metabolically generated from incubation of a mixture of the two oxo-precursors SR121683 and SR121682 with microsomes, four diastereomers being generated from each of them. These two groups of four metabolites could not be discriminated by achiral HPLC, thus leading to identical chromatographic profiles whatever the incubated 2-oxo-enantiomer. On the other hand, using chiral SFC, these metabolites could be differentiated. To validate this hypothesis, we decided to submit separately the two 2-oxo-precursors SR121683 and SR121682 to microsomal biotransformation and the resulting two metabolic pools H were derivatized with acrylonitrile for stabilization. Then, the individual acrylonitrile-derivatized metabolites were analyzed by chiral SFC using a column packed with tris (3,5-dimethylphenyl carbamate) amylose as chiral discriminating agent, which allowed splitting of each HPLC peak into two peaks. Figure 6 shows the chiral SFC chromatogram, thus confirming the existence of 8 distinct stereoisomer (4 enantiomeric pairs) metabolites. This result again is in good agreement with the structure proposal from the MS and NMR studies.

Discussion

Clopidogrel has to be administered *in vivo* to selectively and irreversibly inhibits the binding of 2MeS-ADP to its platelet receptors (Savi et al., 1994a, 2001; Herbert et al., 1999). Clopidogrel is inactive *in vitro* and has to undergo metabolic activation by hepatic cytochrome P450-1A to exhibit its antiaggregating activity (Savi et al., 1992, 1994b). From these studies, a possible metabolic pathway leading to the formation of active metabolite of clopidogrel was tentatively deduced (Savi et al., 2000). In the liver, clopidogrel is metabolized into 2-oxo-clopidogrel through a cytochrome P450-dependent pathway. This intermediate metabolite is then hydrolyzed and generates the highly labile active metabolite, which reacts as a thiol reagent with the ADP receptors on platelets when they pass through the liver. This *in situ* biological effect could account for the absence of an antiaggregating activity in the plasma. In this study, we isolated in sufficient amounts the metabolites of Clopidogrel by incubating the synthetic 2-oxo-clopidogrel with human liver microsomes to determine the chemical structure and biological activity of the active metabolite. The 2-oxo-clopidogrel was used instead of clopidogrel because it has been shown to be generated from clopidogrel by the liver and to show a higher antiaggregating activity *ex vivo* (Savi et al., 1992, 1994b).

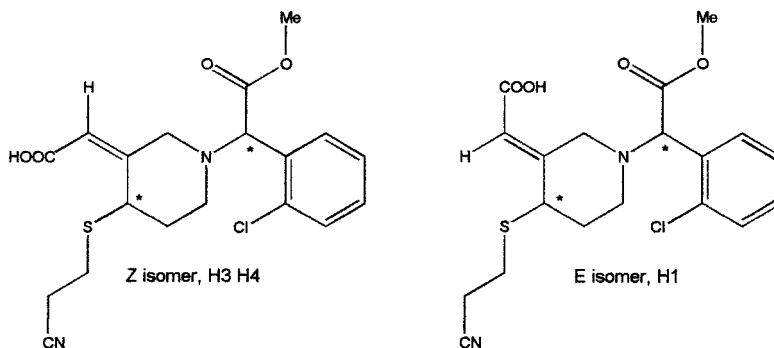


SCHEME 5. 2-[1-[2-(chlorophenyl)-2-methoxy-2-oxoethyl]-4-sulfanyl-3-piperidinylidene]acetic acid.

Incubation of (7S) 2-oxo-clopidogrel with human microsomes led to a pool of metabolites (fraction H), which exhibited a potent *in vitro* activity as assessed by measuring ^{33}P -2-methyl-S-ADP binding to human platelets. This result confirmed the key role played by bio-oxidation of clopidogrel at carbon 2 as an important first step toward the formation of an active metabolite. The active fraction H was shown to be composed of four diastereoisomers only one of which (named H4) with antiplatelet activity. Moreover, parallel microsomal incubations conducted with the inactive (7R) 2-oxo-isomer gave the same HPLC and MS data patterns, whereas no biologically active metabolite could be detected in that case. Altogether, these results underlined the critical importance of a specific absolute stereochemistry and in particular the 7S configuration associated to the active metabolite. We conducted parallel experiments with either the active (7S) or (7R) 2-oxo precursors with a systematic analytical and biological measurement at each step of the purification process. The results indicated that S configuration is preserved in the active fraction since only metabolites issued from the 7S precursor retained biological activity. This strongly suggests that no, even partial, racemization reaction occurs during the incubation with microsomes and/or purification conditions.

The MS data suggested a primary chemical structure with an opened unsaturated thiophene ring, a highly reactive thiol function, and a free carboxylic group. This primary structure was the same for the four H1 to H4 metabolites. This proposed structure bore three stereochemical sites (C 7, C 4, and C 3–C 16 ethylenic bond) and could explain the multiplicity of the observed isomers. The NMR study on the four H metabolic fractions demonstrated that the only active H4 had an ethylenic bond of a Z configuration. Hence, this second stereochemistry factor following the S configuration at carbon 7 was considered to be of crucial importance for the expression of activity. Chiral SFC was able to differentiate eight peaks (two series of four diastereoisomer metabolites), each generated from the active (7S) 2-oxo-clopidogrel or its opposite inactive (7R) 2-oxo-isomer. A diagram showing the possible set of 4 enantiomeric pairs obtained from the (7S) clopidogrel and its opposite (7R) enantiomer is shown in Fig. 7.

The absolute configuration of the stereochemical site C 4 remains the third important structural key to be determined to fully elucidate



SCHEME 4. Z isomer, H3 H4 and E isomer, H1.

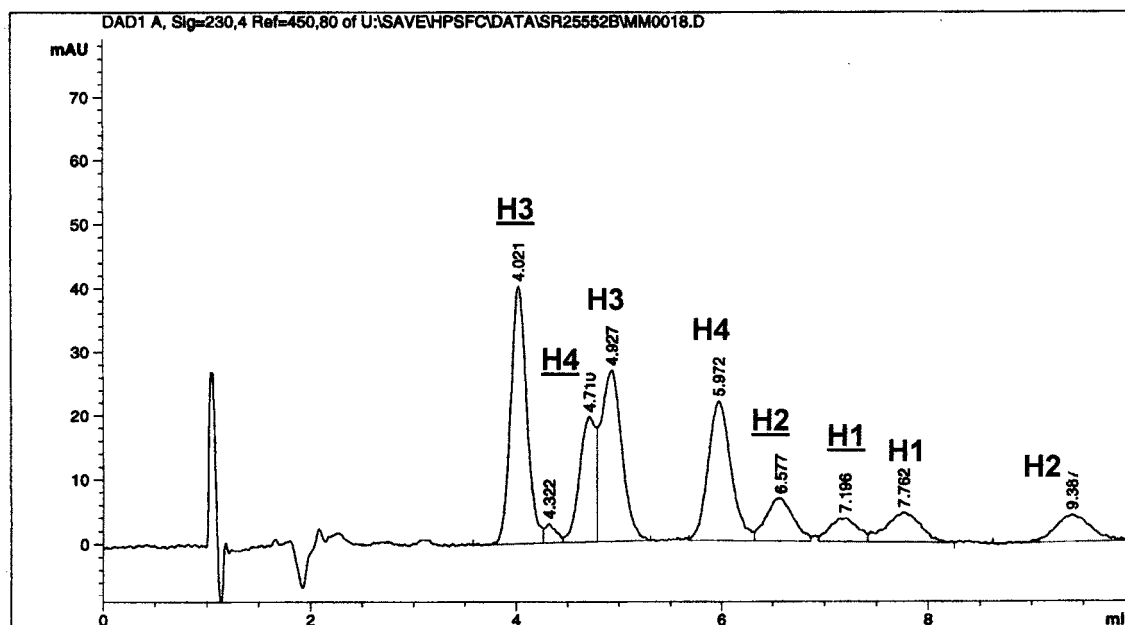


FIG. 6. Chiral SFC chromatogram showing the 4 enantiomeric pairs.

Separation was performed on a reconstituted mixture of four acrylonitrile-derivatized H metabolites from (S) SR121683 and four from (R) SR121682. Underlined label peaks correspond to (S) SR121683 metabolites. Non underlined label peaks correspond to (R) SR121682 metabolites.

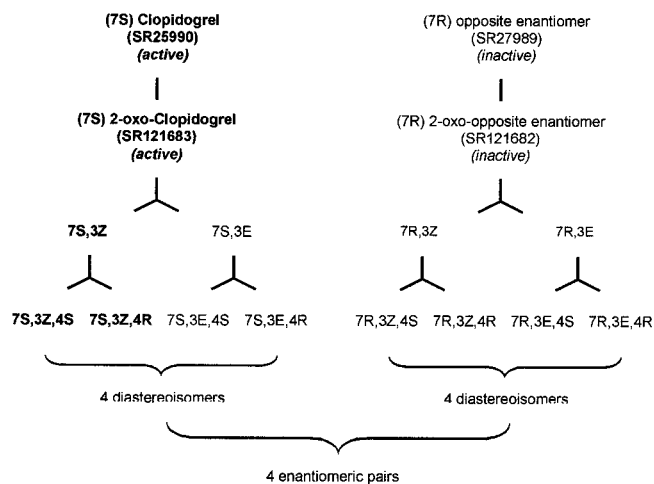


FIG. 7. Possible set of eight stereoisomers generated from Clopidogrel and its opposite inactive SR25989 enantiomer.

the structure of the active H4 metabolite of clopidogrel. Its nature seems to be as important for the activity as the two elucidated configurations since the fraction H3 was shown to have 7S and Z configurations like H4 but was inactive. However, due to the highly unstable character of the active metabolite H4, we have not yet been able to isolate it from human microsomal incubations in sufficient amounts to complete its full characterization by X-ray crystallography.

In conclusion, the present study elucidates the structure and stereochemistry of the active metabolite of clopidogrel generated from human liver microsomes incubated with the 2-oxo-intermediate metabolite. Only one metabolite (bearing 7S, 3Z, and 4S or 4R config-

uration) of the 8 isomers exhibits in vitro the antiaggregating activity of clopidogrel observed ex vivo. This clearly demonstrates that interaction of the active metabolite with its target was highly dependent on its stereochemistry. Whether this compound is the sole active metabolite of clopidogrel remains to be elucidated.

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