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Psorospermin structural requirements for P-glycoprotein resistance reversal

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Abstract

Resistance to chemotherapy reduces its effectiveness, resulting in increased mortality. Psorospermin, a natural product, is a topoisomerase II-directed DNA alkylating agent active against multidrug-resistant (MDR) cell lines, including multiple myeloma. In this study, the mechanism of psorospermin's P-glycoprotein modulation activity and that of its associated pharmacophore were examined. Flow cytometry demonstrates that doxorubicin-resistant multiple myeloma cells (8226/D40) pretreated with psorospermin enhance intracellular retention of doxorubicin, as compared to control (75% versus 38%). Because the overexpression of P-glycoprotein is the primary cause of drug resistance in the 8226/D40 cells, psorospermin-induced sensitization was likely due to *mdr1*/P-glycoprotein expressional or functional inhibition. As shown by PCR and Western blot, neither transcription of *mdr1* nor translation of P-glycoprotein was downregulated by psorospermin treatment. Therefore, the mechanism of psorospermin-induced resistance reversal is most likely through a direct interaction between psorospermin and P-glycoprotein. Furthermore, because only the (2'*R*,3'*R*) isomer of psorospermin showed any resistance reversal activity, the side chain of psorospermin is apparently a crucial moiety for resistance reversal. By understanding the mechanism of psorospermin-induced MDR modulation, psorospermin and similar compounds can be combined with other chemotherapies to treat resistant cancers.

Keywords

psorospermin; P-glycoprotein; topoisomerase II; multidrug-resistance; drug discovery

Introduction

Many cancer patients, especially those diagnosed in the late stages of disease, do not respond to standard treatments. This failure is also frequently observed in patients who relapse after periods of remission (1). Therefore, in order to treat drug-resistant cancers, new chemotherapeutics must be developed.

Cancers resistant to a variety of structurally unrelated chemotherapeutic agents display the MDR phenotype (1,2). One major factor associated with MDR is altered drug transport, namely overactive drug efflux (2,3). Overactive drug efflux prevents drugs from reaching their intended target because transport proteins pump the drug out of the cell in an energy-dependent manner (2–4). The ATP-binding cassette (ABC) protein superfamily has been identified to

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play an integral role in drug efflux and invariably drug resistance. Three ABC proteins frequently overexpressed in many human cancers are P-glycoprotein (P-gp), multidrug resistance-associated protein, and breast cancer resistance protein (5,6).

P-gp, the most studied human ABC transporter, is a 170-kDa dimeric protein, which is the product of the *mdr1* gene (7). Containing two homologous nucleotide-binding domains and two six-membered transmembrane domains, P-gp forms a pore in the cellular membrane, and the interface between the two transmembrane domains is the site for substrate binding (8). Although the mechanism of efflux is not fully understood, studies suggest that substrate binding results in ATP-driven dimerization of the nucleotide-binding domains. This reaction produces a conformation change of the transmembrane domains, leading to a decrease in the binding affinity of the substrate to the binding site, thus releasing the substrate into the extracellular space (8–10). While P-gp functions in normal tissues to remove toxins from the body, it is frequently overexpressed in neoplastic tissues, resulting in the efflux of a variety of structurally unrelated compounds, including chemotherapeutics (5,6,9,11).

Psorospermin was isolated from the stem bark and roots of the African plant *Psorospermum febrifugum* (12,13). Although studied for over thirty years, the four isomers of psorospermin were only recently synthesized in 2005 (Figure 1A) (14). Significantly, psorospermin has activity against drug-resistant cell lines, including breast cancer, leukemias, and AIDS-related lymphomas (15–17). Psorospermin's cytotoxic activity stems from its ability to intercalate with DNA at the major groove by site-directed electrophilic addition, resulting in a covalent psorospermin–DNA adduct (Figure 1B) (18). We previously have shown that the alkylation activity of psorospermin is greatly increased in the presence of topoisomerase II, an enzyme that assists in DNA breakage and re-ligation. This increase is due to psorospermin's ability to trap the topoisomerase II–DNA complex in the initial noncovalent binding step of the topoisomerase II catalytic cycle, resulting in topoisomerase II–mediated DNA cleavage (19). Due to its mechanism of topoisomerase II–directed alkylation, psorospermin has been characterized as a novel topoisomerase II poison (18,19).

Because psorospermin's cytotoxic activity is not affected by overexpression of P-gp or other ABC transport proteins (17), psorospermin may play a role in MDR modulation. In this study, we investigate the mechanism underlying psorospermin's P-gp modulation activity in multiple myeloma cells. Our studies have shown that treatment with the natural isoform (2'R,3'R), and not the other isoforms, of psorospermin inhibits the doxorubicin export in these cells. Furthermore, we observed that neither *mdr1* transcription nor P-gp translation is inhibited by psorospermin, leading to the hypothesis that psorospermin affects P-gp directly.

Materials and Methods

Cell Culture and Compounds

8226/S (parental) human multiple myeloma cells were purchased from ATCC (Manassas, VA). The doxorubicin-resistant 8226 cell line (8226/D40) was a gift from the Dalton laboratory (University of Arizona, Tucson, AZ) (20). Both 8226/S and 8226/D40 cell lines were cultured in RPMI 1640 media (CellGro, Manassas, VA) supplemented with 8% fetal bovine serum (Gemini, West Sacramento, CA) and 1% Penicillin/Streptomycin (CellGro). Cells were maintained at 37 °C in a humidified incubator at 5% CO₂. Cells were fed with media every 2 to 3 days and passaged weekly. Doxorubicin was purchased from Fluka Biochemika (Buchs, Switzerland) and diluted in water to a stock concentration of 1.8 mM. (±)-Verapamil hydrochloride was purchased from Fluka Biochemika and was diluted to 2.2 mM in 100% methanol. All four isomers of psorospermin (PS-RR, PS-RS, PS-SR, and PS-SS) were obtained from Cylene Pharmaceuticals (San Diego, CA) and diluted in DMSO to a stock concentration of 10 mM. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-

tetrazolium, inner salt (MTS), and phenazine ethosulfate were purchased from Promega (Madison, WI). P-gp antibody was purchased from Abcam (Cambridge, MA). The goat anti-mouse IgG-HRP conjugated antibody was purchased from BioRad (Hercules, CA).

Doxorubicin Accumulation Assay

8226/S and 8226/D40 cells were plated at a concentration of 1×10^6 cells/mL in 250 mL conical tubes. The cells were pretreated with 5 μ M psorospermin, 13 μ M verapamil, or were not pretreated. After 15 minutes, all cells were treated with 10 μ M doxorubicin and were incubated at 37 °C for 1 hour. The reaction was quenched with ice-cold PBS, and the cells were pelleted by centrifugation (2000 rpm, 2 minutes). The cells were resuspended at a concentration of 2.5×10^5 cells/mL in media with or without psorospermin or verapamil. The cells were incubated at 37 °C. At time 0 and after 60 minutes, aliquots of 1×10^6 cells were removed from each sample in triplicate. The reaction was quenched with ice-cold PBS, and the cells were pelleted by centrifugation. The cells were resuspended in ice-cold PBS, and intracellular doxorubicin content was quantitated by a FacScan flow cytometer (BD Biosciences, Franklin Lakes, NJ) at 488ex/585em nm.

Cell Viability Assay

Cells were plated at a concentration of 5×10^3 cells/well in a 96-well plate and treated with drug at indicated concentrations. The cells were incubated at 37 °C for 3 days. Cell viability was measured using a CellTiter 96® AQueous One Solution Cell Proliferation Kit (Promega, Madison, WI). Briefly, MTS and PMS were combined at a 20:1 ratio, and 20 μ L was added to each well. The cells were incubated for 3.5 hours, and absorbance was read at 490 nm using a plate reader.

Transcription and Translation of *mdr1*/P-glycoprotein

In the *mdr1* transcription experiments, cells were plated at a concentration of 2×10^6 cells in T75 flasks. The cells were treated with (2'R,3'R) psorospermin (PS-RR) at concentrations of 2.5 and 5 μ M. DMSO was used as a vehicle control. After incubating the cells at 37 °C for 2 hours, the cells were pelleted and washed with ice-cold PBS. Total RNA was extracted using the Nucleospin RNA II kit (Macherey-Nagel, Bethlehem, PA) and quantitated by spectroscopy (260 nm). Complementary DNA was made with 1 μ g RNA using the iScript cDNA Synthesis Kit (BioRad). For use in real-time PCR, primers for *mdr1* were designed using MacVector (MacVector, Inc., Cary, NC). The primer sequences used were 5'-CATTGGTGTGGTGAGTCAGG-3' (forward) and 5'-CATAGGCATTGGCTTCCTTG-3' (reverse). β -actin was used as a housekeeping gene. Real-time PCR was performed using the BioRad MyiQ Single-Color Real-Time PCR Detection System with SYBR green 1 fluorophore (BioRad). The BioRad protocol was followed with an optimized annealing temperature of 59 °C.

To measure the protein expression of P-gp, 2×10^8 cells treated with PS-RR for 2 hours (as above) were lysed and fractionated as follows. Cells were resuspended in Hypotonic Lysis Buffer (10 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH 7.4, Protease Inhibitor Tablet (Roche)) and ruptured with a 15 mL homogenizer (25 strokes). Lysate was centrifuged at 5,700 rpm, 4 °C, for 10 minutes. The supernatant was centrifuged at 25,000 rpm, 4 °C, for 1.5 hours and the pellet was frozen at -80 °C overnight. Pellet was resuspended in a 1:1 mixture of 5 mM Tris-HCl (pH 7.4) and 16% sucrose and homogenized using a 7 mL homogenizer. The lysate/sucrose mixture was added to the top of a 16–60% non-continuous sucrose gradient and centrifuged at 27,000 rpm, 4 °C, for 4 hours. The membrane vesicles were collected from the 16–31% and 31–45% interfaces, diluted with 5 mM Tris-HCl (pH 7.4), and centrifuged at 27,000 rpm, 4 °C, for 1 hour. The membrane pellet was resuspended in 5 mM Tris-HCl (pH 7.4) (25 μ L) and the protein concentration was measured by BCA assay (BioRad). 30 μ g total

protein were separated by SDS-PAGE using a 4–20% Tris-HCl gel. The protein was transferred to nitrocellulose using a BioRad Mini Trans-Blot® Electrophoretic Transfer Cell (6V for 20 minutes followed by 20V for 3 hours). After blocking the filter with 5% non-fat dry milk/TBST (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween-20), the membrane was agitated in blocking buffer with P-gp primary antibody (1:1000) for 2 hours at room temperature. After washing the membrane with TBST, it was agitated for 1 hour in blocking buffer with the goat anti-mouse secondary antibody (1:1000). The membrane was again washed with TBST and stained with chemoluminescent solution (Pierce, Rockford, IL). The membrane was exposed to film and developed. Using the ImageJ software (National Institutes of Health, Bethesda, MD), Optical Density analysis was used to quantitate changes in P-gp expression.

Psorospermin Superposition Molecular Modeling

Psorospermin, fluphenazine, and GF-120918 were constructed using the Insight II Builder program (Insight II 2005L, Molecular Modeling Software, Accelrys Inc., 9685 Scranton Rd., San Diego, CA 92121). Molecules were then minimized using the Discover 3.0 minimization program within the Insight II software, and the minimized energy structures were used for superimposition. The psorospermin molecule was superimposed with fluphenazine and GF-120918 using structural similarity and manual superimposition.

Results

Psorospermin can induce drug-resistance reversal in multiple myeloma

Previous data demonstrated equivalent psorospermin cytotoxicity in the 8226/S and 8226/D40 cell lines. In comparison, in the same pair of cell lines, doxorubicin showed a 500-fold level of resistance (17). These data indicate that psorospermin is either not a substrate for P-gp or perhaps a modulator of it.

To measure the P-gp reversal activity of psorospermin, doxorubicin efflux assays were performed, and relative intracellular doxorubicin concentrations were measured by flow cytometry (Figure 2). The 8226/D40 cells that were only treated with doxorubicin (no psorospermin or verapamil) showed a significant decrease in intracellular doxorubicin after 60 minutes of efflux, retaining only 38.2% of the initial doxorubicin. However, 8226/D40 cells that were treated with PS-RR retained the majority (74.7%) of the doxorubicin after 60 minutes. As a comparison, doxorubicin efflux in the presence of a known P-gp modulator, verapamil, was greatly inhibited: 82.2% of doxorubicin was retained in the same time interval. The 8226/S cells did not show any significant change in doxorubicin levels over 60 minutes of efflux with or without psorospermin or verapamil (data not shown).

The dose dependency and mechanism of psorospermin-induced resistance reversal are different from those producing the cytotoxicity

To identify a psorospermin concentration at which only chemosensitization, and minimal cytotoxicity (< 10%), is observed, PS-RR dose response curves were generated using MTS assays (data not shown). 3 nM PS-RR produced a decrease in cell viability of 10.2% in the 8226/D40 cells. Therefore, using three psorospermin doses (1, 5, and 10 nM), the enhancement of doxorubicin cytotoxicity in the presence of psorospermin was examined. Since the 8226/D40 cell line had a 500-fold level of resistance to doxorubicin, a 4-log concentration differential was used, with a typical exposure period of three days. When psorospermin is combined with doxorubicin, even at very low concentrations (5 nM and 10 nM), the cytotoxic effect overwhelmed the reversal effect (Figure 3). Thus, the data indicate that the percent survival is reduced to about 50% and 20% for those two psorospermin concentrations. Then, as the doxorubicin concentration increases, doxorubicin's cytotoxic effect is observed, but this appears to be an additive effect with psorospermin. At the lowest concentration of PS-RR (1

nM), no effect is seen. The reversal effect (Figure 2) requires a higher concentration (5 μ M) and shorter exposure time (1 h) than those of the PS alkylation-mediated cytotoxic effect (5–10 nM and three days). This is a reflection of the short time but large dose required to induce P-gp versus the long time but small dose of psorospermin required to produce the cytotoxic effects.

Psorospermin does not significantly alter *mdr1*/P-glycoprotein transcription or translation

To determine if the P-gp reversal by psorospermin can be attributed to transcription or translation of the *mdr1* gene or P-gp, respectively, these endpoints were measured following treatment with psorospermin. Because resistance reversal has been observed within 2 hours after psorospermin treatment (Figure 2), it was important to determine how the *mdr1* mRNA and P-gp expression levels changed within that time period. Therefore, using the 8226/S and 8226/D40 cell lines, the cells were treated with PS-RR at concentrations of 0 (vehicle), 2.5, and 5 μ M for 2 hours. The cells were then collected and processed for real-time PCR and Western blot analysis. The transcriptional analysis showed that the *mdr1* mRNA levels did not change significantly in either cell line when treated with PS-RR (Figure 4A). When P-gp from PS-RR-treated membrane isolates was measured, an increase of 23.1% and 51.4% (as determined by Optical Density analysis) was observed in 8226/D40 cells treated with 2.5 μ M and 5 μ M PS-RR, respectively, compared to vehicle control (Figure 4B). As expected, no P-gp was detected in the 8226/S cells, which do not overexpress P-gp (20).

The resistance reversal activity of psorospermin is stereospecific

The cytotoxicity of psorospermin varies based on its stereochemistry. Previous studies have determined that, in the 8226/D40 cell line, the IC_{50} of psorospermin ranges from 0.037 μ M (PS-RR) to 0.335 μ M (PS-SS) (17). Due to this variability of cytotoxic potency, we determined if stereochemistry was a factor in its resistance-reversal activity. By doxorubicin accumulation assay, 8226/D40 cells that were treated with the PS-RR isomer demonstrated a significant retention (80.4%) of doxorubicin after 60 minutes compared with cells treated with doxorubicin only (36.2%), similar to that seen in Figure 2. As previously reported, verapamil retained doxorubicin at a comparable level (Figure 5). The other three isomers of psorospermin (PS-RS, PS-SR, and PS-SS) did not show any significant retention of doxorubicin compared to the control.

Discussion

In this study, we have achieved a better understanding of the mechanism of psorospermin-induced P-gp resistance reversal. Psorospermin's resistance reversal activity appears to be due to its stereospecific interaction with P-gp directly and is not based on transcriptional or translational downregulation.

Previous studies have shown that psorospermin has cytotoxic activity in drug-resistant cancers, including doxorubicin-resistant breast cancer and multiple myeloma (17). This cytotoxicity has been attributed to its interaction with topoisomerase II and DNA (19). However, currently, treatment of doxorubicin-resistant 8226 cells with PS-RR has resulted in a significantly enhanced intracellular retention of doxorubicin. Since the doxorubicin resistance of the 8226/D40 cell line is due to an overexpression of P-gp (17,20), psorospermin-induced resistance reversal may be attributed to an inhibition of P-gp function. By reducing P-gp activity, doxorubicin efflux is decreased, allowing the drug to accumulate intracellularly.

Although it is apparent that psorospermin plays a critical role in the observed doxorubicin retention, psorospermin's mechanism of P-gp inhibition is still unclear. Because inhibition of transcriptional and/or translational activity of *mdr1*/P-gp and inhibition by a direct interaction

with the protein could both result in a decrease of drug efflux, each possible mechanism had to be examined. Treatment with PS-RR did not result in a decrease in either *mdr1* transcription or P-gp translation within the time interval in which resistance reversal was observed.

Therefore, it is unlikely that psorospermin-induced resistance reversal is due to transcriptional/translational regulation. In particular, psorospermin (5 μ M) increased P-gp protein expression in the 8226/D40 cells, yet P-gp function was decreased, further highlighting the likely physical interaction of PS-RR with P-gp. The observed increase in P-gp expression in the presence of PS-RR may be indicative of an attempt by the cells to counteract the resistance-reversal effect of PS-RR. However, the hypothesis that transcriptional/translational regulation is not involved in resistance-reversal is still strengthened by the fact that psorospermin-induced resistance-reversal is observed within two hours of treatment, while the half-lives of the *mdr1* mRNA and P-gp are 4–10 hours and 14–72 hours in P-gp-overexpressing cell lines, respectively (21–24), indicating that psorospermin would most likely have to interact directly with the P-gp to inhibit drug efflux, as observed.

Because a direct interaction between psorospermin and P-gp appears to be necessary for resistance reversal activity, it was not surprising that the stereochemistry of psorospermin significantly affects potency. Because only PS-RR was shown to have any resistance reversal activity, this activity is stereospecific. The two chiral centers of psorospermin, which define the four diastereomers, are on the side chain of the molecule and not the xanthone ring. Thus, the side chain is apparently necessary for P-gp functional inhibition. While the side chain is necessary for any inhibitory activity, the xanthone ring is also likely to be functionally relevant. Established P-gp modulators, such as fluphenazine (a first-generation MDR modulator) and GF-120918 (a third-generation MDR modulator), contain planar ring structures that are similar to the xanthone ring of psorospermin. An overlay of these molecules and psorospermin showed that the xanthone ring of psorospermin fits well with the phenothiazine ring of fluphenazine (RMS value of 0.221) (Figure 6A) and the 9-acridinol ring of GF-120918 (RMS value of 0.09) (Figure 6B). Because fluphenazine and GF-120918 have been shown to have in vitro reversal activity in drug-resistant solid cancers, psorospermin, a compound with a similar structure, could be expected to display resistance-reversal activity as well. It is possible that the planar xanthone ring is necessary to allow for psorospermin to access and interact with the binding pocket of P-gp.

Previous studies by Seelig and colleagues (25,26) that identified general structural elements necessary for P-gp substrate recognition correlate with our psorospermin findings. Seelig proposed that substrates of P-gp must contain at least one type I (two electron donor groups separated by 2.5 ± 0.3 Å) or one type II (two or three electron donor groups separated by 4.6 ± 0.6 Å (outer groups)) electron donor group to interact with P-gp. Using these criteria, it is not surprising that psorospermin may interact with P-gp because its structure contains two type I electron donor groups (a carbonyl and ether group separated by 2.71 Å and an alcohol and an ether separated by 2.77 Å (Figure 6C)), characteristic of a P-gp substrate. These structural elements are comparable to that of verapamil, an established resistance reversal agent (27, 28) that has two type I electron donor units but also one type II unit (26). Although psorospermin's mechanism of resistance reversal is not yet known, but because both psorospermin and verapamil contain multiple type I electron donor groups and also display similar levels of resistance reversal activity, psorospermin is likely to share a similar mechanism with verapamil, which competitively inhibits P-gp (29). If this is the case, psorospermin would act as a competitive inhibitor of P-gp, thus reversing the cell's resistance mechanism and preventing efflux of doxorubicin and allowing doxorubicin to interact with topoisomerase II. This illustrates the unique dual mechanism of psorospermin. Psorospermin can act both by a topoisomerase II-mediated alkylation mechanism as well as by a specific mechanism that inhibits P-gp and causes resistance reversal. The stereochemistry required for

both mechanisms is the same (2'*R*,3'*R*) and corresponds to the natural isomer of psorospermin. Taken together, this data shows how important stereochemistry is for psorospermin activity.

Psorospermin is able to produce two distinct effects: topoisomerase II-directed DNA alkylation and P-gp-mediated resistance, which are both relevant to the ability of psorospermin to kill cells that overexpress P-gp, the dose required to reverse doxorubicin resistance is about 1000 times higher than that required for the cytotoxic effect of psorospermin. This is really a reflection of the comparative cytotoxicities of the two drugs. The only way psorospermin could be used to reverse doxorubicin cytotoxicity would be to eliminate the alkylation ability of psorospermin. Nevertheless, the structural insight into the P-gp resistance reversal by psorospermin is an important step toward this goal.

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Abbreviations

ABC	ATP-binding cassette
Dox	doxorubicin
MDR	multidrug-resistance/resistant
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
P-gp	

	P-glycoprotein
PS-RR	2' <i>R</i> ,3' <i>R</i> Psorospermin
PS-RS	2' <i>R</i> ,3' <i>S</i> Psorospermin
PS-SR	2' <i>S</i> ,3' <i>R</i> Psorospermin
PS-SS	2' <i>S</i> ,3' <i>S</i> Psorospermin
RMS	root mean square deviation
Vera	verapamil

Figure 1.

(A) Structures of the four isomers of psorospermin. (B) Covalent alkylation reaction between psorospermin and N7 of guanine of DNA (reproduced from ref. 18).

Figure 2. Intracellular doxorubicin fluorescence in 8226/D40 cells treated with psorospermin (PS-RR) or verapamil. Doxorubicin (10 μ M) fluorescence was measured after 60 minutes of uptake (0 min) and following efflux (60 min) in the absence or presence of verapamil (13 μ M) or PS-RR (5 μ M) using flow cytometric analysis. Doxorubicin fluorescence was normalized to the measurement at time 0.

Figure 3.

Doxorubicin dose response curve of 8226/D40 cells treated with PS-RR or verapamil. Cells were pretreated with vehicle, PS-RR, or verapamil and increasing concentrations of doxorubicin. After three days, cell viability was measure by MTS assay.

Figure 4.

(A) Mdr1 gene expression in 8226/S and 8226/D40 cells treated with PS-RR. Cells were treated with DMSO, 2.5, and 5 μM PS-RR for two hours. Mdr1 mRNA levels were quantitated by real-time PCR. The measurements were normalized to the untreated control. (B) P-gp protein levels of membrane isolates from 8226/S and 8226/D40 cells treated with PS-RR for two hours. Cells were treated as above. Membranes were isolated and P-gp levels were measured by Western blot.

Figure 5.

Intracellular doxorubicin fluorescence in 8226/D40 cells treated with psorospermin isomers or verapamil. Cells were pretreated with 5 μ M PS-RR, PS-RS, PS-SR, or PS-SS or 13 μ M verapamil, and doxorubicin uptake and efflux were examined. Doxorubicin fluorescence was normalized to the measurement at efflux time 0.

Figure 6.

Superimposition of psorospermin and established P-gp modulators. Using the Insight II Builder program and its Discover 3.0 minimization program, the molecules' minimized energy structures were used to construct an overlay of psorospermin (colored by atoms) with (A) fluphenazine (red) and (B) GF-120918 (red). (C) Identification of the type I electron donor groups of PS-RR. Measurements were made between oxygen atoms.