Bacterial spores are remarkable in their resistance to chemical and physical stresses, including exposure to UV radiation. The unusual UV resistance of bacterial spores is a result of the unique photochemistry of spore DNA coupled with the efficient repair of accumulated damage. Exposure of bacterial spores to UV radiation results in the formation of a methylene-bridged thymine dimer, 5-thyminyl-5,6-dihydrothymine (spore photoproduct, or SP), as the primary photoproduct.\(^1\,^3\) SP accumulates in UV-irradiated spores; however, it is rapidly repaired upon germination, thus giving rise to the extraordinary UV resistance of bacterial spores.\(^4\,^5\) The repair of SP is catalyzed by the enzyme spore photoproduct lyase (SPL), and involves the direct reversal of SP to two thymines without base excision (Scheme 1).

SPL is a member of the radical AdoMet superfamily, and utilizes a [4Fe–4S] cluster and S-adenosylmethionine (AdoMet) as essential cofactors in SP repair.\(^6\,^9\) We have previously shown that SP repair is initiated by abstraction of H\(^+\) from C6 of SP by an AdoMet-derived S'-deoxyadenosyl radical;\(^10\,^11\) this H-atom abstraction is thought to initiate a radical-mediated \(\beta\)-scission of the C5–C\(_{\text{bridge}}\) bond in the photoproduct, as originally proposed by Mehl and Begley.\(^12\)

While two distinct diastereomers of SP (5\(R\) or 5\(S\), Figure 1) could in principle be formed upon UV irradiation of bacterial spores, only the 5\(R\) configuration is possible for SP formed from adjacent thymines in double helical DNA, due to the constraints imposed by the DNA structure.\(^13\,\14\) The 5\(S\) configuration, therefore, is possible only in less well-defined DNA structures or as an interstrand crosslink. It was thus quite surprising when two recent reports concluded that SPL repairs only the 5\(S\), and not the 5\(R\), isomer of a synthetic SP substrate.\(^14\,\15\) We report here results from HPLC and MS analysis of \textit{in vitro} enzymatic assays on stereochemically defined synthetic SP substrates demonstrating that SPL specifically repairs only the 5\(R\) isomer of SP. This stereospecific repair of 5\(R\)-SP by SPL is consistent with the longstanding hypothesis that SP is a result of UV-induced dimerization of adjacent thymines in double-helical DNA.

SPL was cloned from \textit{Clostridium acetobutylicum}, overexpressed in \textit{Escherichia coli}, and purified using a method similar to published procedures.\(^11\) The enzyme contained 2.9 (±0.2) Fe per SPL, and had UV–visible and EPR spectroscopic properties characteristic of an iron–sulfur enzyme. The 5\(R\) and 5\(S\) diastereomers of protected (N-SEM, O-TES, and O-TBDMS) SP were synthesized using modifications of published procedures,\(^13\,\15\) and were subsequently deprotected (Supporting Information (SI)). The structures of the fully protected, the di-SEM protected, and fully deprotected dinucleoside spore products were confirmed by \(^1H\) and \(^{13}\)C NMR techniques, and NOESY and ROESY were used to assign the stereochemistry at C-5 (SI). To remove any potential ambiguity associated with the assignment of stereochemistry at C-5 in the open dinucleoside forms of SP, the protected open dinucleosides (5\(R\) and 5\(S\)) were converted to closed cyclic phosphotriesters, and the stereochemical assignments at C-5 were confirmed by NOESY and ROESY (SI). Stereochemical assignments were based on initially assigning the pro-\(S\) and pro-\(R\) hydrogens at C6, with the pro-\(R\) hydrogen appearing downfield of the pro-\(S\) hydrogen for nearly all of the synthetic forms of both diastereomers of SP (SI). Our assignment of pro-\(R\) and pro-\(S\) hydrogens differs from that reported previously, and may account for the disparity between our results and those previously published.\(^14\,\15\) Proper assignment of the pro-\(R\) and pro-\(S\) hydrogens is critical to the determination of stereochemistry at C5, as the coupling between these protons and the C5 methyl, as examined by 2D NOESY and ROESY, can then be used to assign the absolute configuration. Our assignments of stereochemistry are completely consistent with distances derived from our computational models of the two diastereomers of SP (SI).

**Scheme 1.** Formation and Repair of Spore Photoproduct

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Figure 1. Chemical drawings (top) and computational models (bottom) for the 5\(R\) (left) and 5\(S\) (right) synthetic spore photoproduct analogues lacking a DNA backbone.
in Figure 2 have allowed us to quantify the rate of SP repair in this
manner. The synthetic SP is not
comparable to the synthetic SP relative to SP generated by UV irradiation
and/or to enzyme inactivation. The lower rate of turnover for
Figure 2C, likely due to enzyme instability under assay conditions,
and/or to enzyme inactivation. The lower rate of turnover for
Figure 2C under anaerobic
conditions; details are provided in SI. Under the HPLC conditions
utilized 5R-SP elutes at 19.3 min, 5S-SP elutes at 20.3 min, and
thyminde elutes at 14.4 min under these conditions. The identity of this emerging peak as thyminde was confirmed by
co-injection with authentic thyminde and by MS analysis of HPLC
fractions corresponding to the peak (SI). The synthetic SP as well
as the SP peaks in Figure 2 (m/z = 485 (SP) and 507 (SP + Na+)),
as well as the peak labeled T in Figure 2 (m/z = 265 (T + Na+)),
gave rise to the expected pattern upon MS analysis. MS analysis of the small peaks eluting between 15 and 17 min show them to result from inhomogenous mixture of small peptides, presumably resulting from protein degradation during the sample workup. These assays have been performed seven different times, on two different
protein samples, using two different buffers and two different reducing agents (dithionite or 5-deazariboflavin), and in all cases
SPL repaired the 5R but not the 5S-SP.
Integration of the thyminde and SP peaks in the chromatograms
in Figure 2 have allowed us to quantify the rate of SP repair in this
system (Figure 2C) as ~0.4 nmol/min/mg of SPL for the R isomer,
and 0 nmol/min/mg of SPL for the S isomer. These rates can be compared to the rate of 0.33 μmol/min/mg when using B. subtilis
SPL and UV-irradiated plasmid DNA as a substrate. Little
additional SP turnover occurs after the 4 hour time point shown in
Figure 2C, likely due to enzyme instability under assay conditions,
and/or to enzyme inactivation. The lower rate of turnover for
synthetic dinucleoside SP relative to SP generated by UV irradiation
of plasmid DNA is not surprising, as the synthetic SP is not
constrained to the conformation found in a DNA strand, and also
lacks the phosphodiester bridge and oligonucleotide strand that
likely contribute considerably to substrate binding interactions in the
SPL active site. We have incorporated the protected SP
phosphodiester described in the Supporting Information into an
oligo; however, our initial assays on this indicated that the enzyme
cannot repair the di-SEM protected SP analogue. Carell has also
incorporated a synthetic SP analogue into an oligonucleotide strand,
however the analogue contains no phosphodiester linkage; no assay
data on the resulting oligos were presented.16 We are currently
pursuing deprotection of the SP phosphodiester (SI) and incor-
poration of them into oligonucleotide DNA, to produce stereo-
chemically defined substrates for further assay and mechanistic
studies.

The results presented herein provide direct and quantitative
evidence for the stereochemical requirements of SP repair by SPL,
demonstrating that SP repairs specifically the 5R isomer of SP.
The identification of 5R-SP as the substrate of SPL supports the
premise that SP, like the more familiar cyclobutane thymine dimer,
is a result of UV-induced dimerization of adjacent thymines in a
DNA strand. Furthermore, the observation that only 5R-SP is a
substrate for SPL is consistent with the expectation, based on the
constraints imposed by the DNA double helical structure, that 5R
is the SP isomer produced in vivo upon UV irradiation of bacterial
spore DNA, an expectation supported by a recent report showing
that 5R SP is the diastereomer produced upon UV irradiation of a
TpT dinucleotide.17

Acknowledgment. The authors thank the National Institutes of
Health for financial support of this research (GM67804). We gratefully acknowledge the assistance of Valerie Copie on computational modeling of SP. Funds to purchase the Bruker 600 MHz NMR were provided by the NIH (SIG 1-S10RR13878) and the NSF (EPSCOR- Montana).

Supporting Information Available: Synthetic methods and NMR
colorization of all synthetic SPs mentioned herein; NOESY and
ROESY of selected compounds; molecular modeling of SPs; mass spec
data on T and SP peaks from the HPLC-based assay. This material is
available free of charge via the Internet at http://pubs.acs.org.

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JA807375C