Direct repair of a synthetic 5S-configured spore photoproduct by a spore photoproduct lyase.

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The DNA-Repair Enzyme "Spore Photoproduct Lyase" Repairs The Interstrand 5*S*-Configured Spore Photoproduct**

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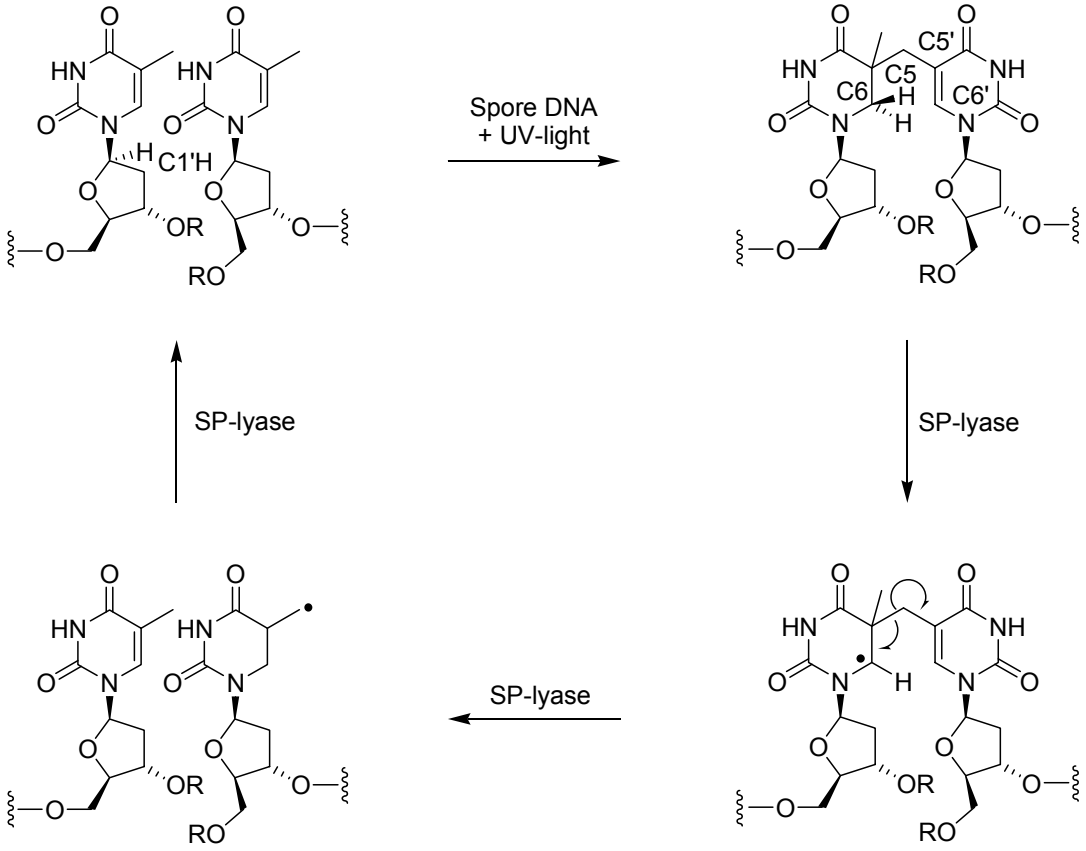
Bacteria of the *Bacillus* and *Clostridium* species form metabolically dormant endospores in response to nutrient depletion. Spores are entirely different compared to the vegetative cell, which allows them to be resistant to conditions such as toxic chemicals, heat or desiccation. Spores are in addition stable over extreme periods of time.[1] One of the most striking feature is their 50 fold increased resistance to 254 nm UV-light.[2] UV irradiation of cells induces in DNA the formation of a variety of mutagenic UV lesions such as cyclobutane pyrimidine dimers[3] and (6-4) photoadducts.[4] The DNA in spores, however, has a very different UV-photochemistry.[5, 6] Only small amounts of the standard DNA lesions are formed. Instead the irradiation gives rise to a unique spore photoproduct lesion.[1] The completely different reactivity is currently explained with the unusual packing of DNA in spores, which seems to allow storage of the genetic information even under harshest conditions over time. The DNA in spores is strongly dehydrated and tightly bound to small acid soluble proteins (SASP's).[7, 8] These conditions seem to induce an unusual DNA structure, which upon UV-irradiation gives rise to this novel, for the spore lethal, spore photoproduct shown in Scheme 1.[9, 10]



Scheme 1. Formation and repair of the spore photoproduct DNA lesion in UV-irradiated spores. R: It is today unknown whether the lesion is a crosslink (R = H) or an intrastrand lesion with R than being a central phosphodiester group as in standard DNA. SP-lyase = spore photoproduct lyase.

Today neither the mechanism of spore photoproduct formation nor the stereochemistry at C5 (see Scheme 1 for numbering) of the spore DNA lesion is known. In addition, it is at this point not clear if the spore photoproduct is formed in an intrastrand reaction, which would produce a lesion with a central phosphodiester group or if it exists as an interstrand crosslink. The latter situation would give a lesion which lacks the phosphordiester group. Recently Cadet and Douki concluded, based on HPLC-MS/MS data obtained from irradiated and fully digested dehydrated model DNA, that the spore photoproduct may be formed in a significant yield as an interstrand crosslink lesion.[11]

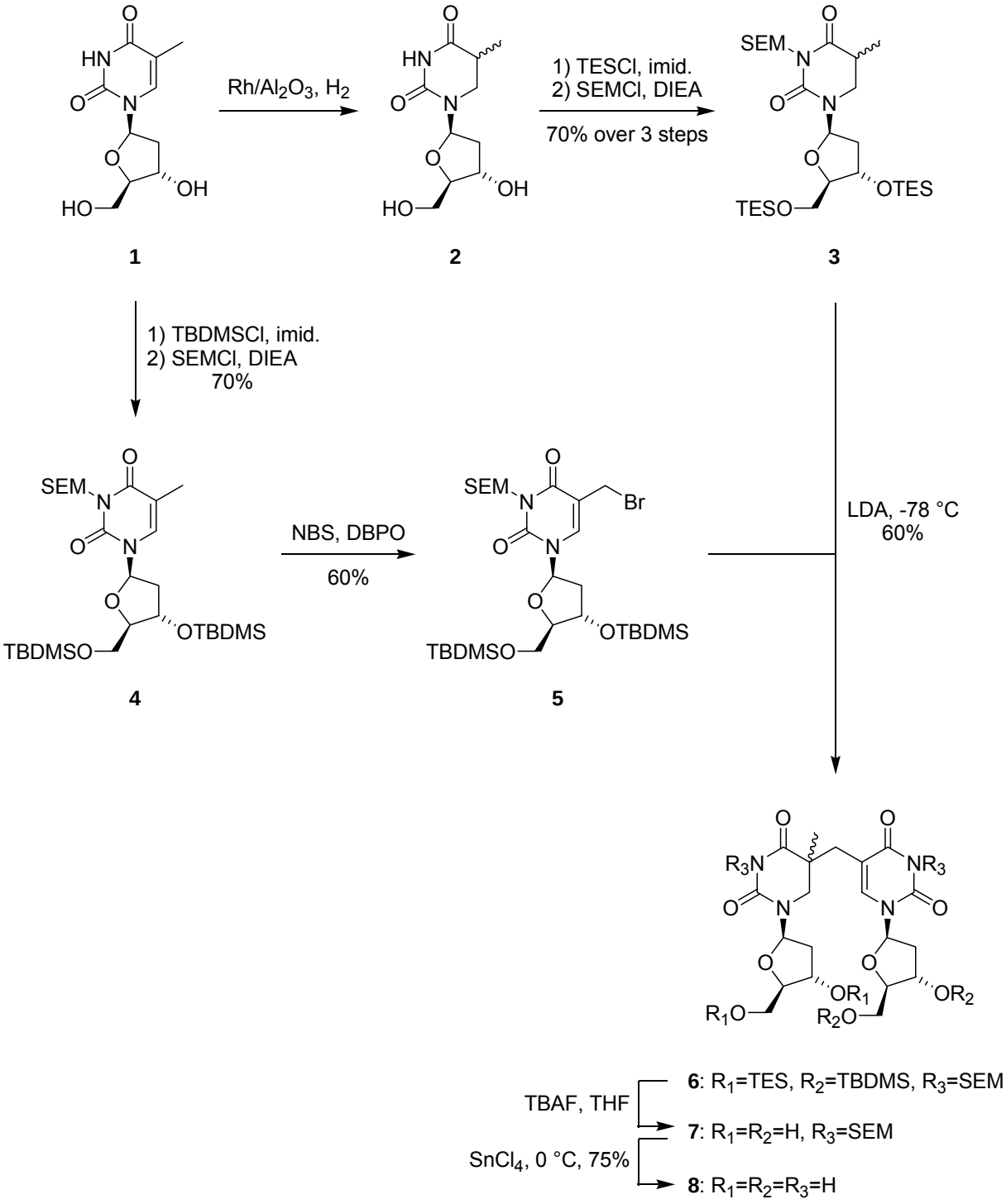
Revival of spores requires efficient repair of the unusual spore photo product. Repair is performed with an enigmatic repair enzyme called spore photoproduct lyase (SP-lyase).[12-15] Sequence comparisons, preliminary spectroscopic studies, and a recent labelling experiment provided evidence that the SP-lyase is a member of the radical-SAM enzyme family.[16] SP-lyase thus is a (4Fe-4S) protein catalyzing lesion repair as depicted in Scheme 1, using a radical based mechanism dependent on S-adenosylmethionine (SAM) as an essential cofactor. [17-20]

So far most of the spore repair investigations were performed with crude DNA substrate obtained by direct irradiation of DNA in the presence of SASP's. In order to gain understanding of the lesion structure, its mutagenicity and repair it is however essential to synthesize the spore photoproduct lesion both in an intrastrand and interstrand version and to analyze repair with the SP-lyase.[21]

Herein we report the synthesis of the interstrand crosslink version of the spore lesion. The successful synthesis allowed us to perform the first repair experiment with the SP-lyase enzyme using defined substrates. This approach allowed us to clarify that the crosslink version is indeed a substrate for the enzyme. We show in addition, that the enzyme recognizes specifically the 5*S*-isomer. This is a surprise because previously it was assumed that the dehydrated DNA in spores has an A-like structure,

and that the steric restrictions imposed by this duplex conformation would favour formation of the *5R*-isomer.[22]

The synthesis was achieved based on earlier work reported by Begley.[22] The synthesis depicted in Scheme 2 starts with thymidine **1**, which is first hydrogenated to give dihydrothymidine **2**. Protection of the hydroxyl groups and of the ring imide was performed with triethylsilylchloride (TES-Cl) and 2-(trimethylsilyl)ethoxymethylchloride (SEM-Cl) to give compound **3**. A second batch of thymidine was *tert*-butyldimethylsilyl- (TBDMS) and SEM-protected to **4** and subsequently converted into the bromide **5**. Deprotonation of the dihydrothymidine compound **3** with LDA and coupling of the enolate with the allylbromide **5** afforded the methyl linked *bis*-thymidine compound in form of two diastereoisomers (**6a** + **6b**). Cleavage of the TES and TBDMS groups with tetrabutylammoniumfluoride (TBAF) furnished the SEM-protected diastereoisomers **7a** and **7b**. Selective cleavage of the TES and TBDMS groups is also possible, which is important for the future synthesis of a phosphoramidite building block required for DNA synthesis. On the stage of **7a** / **7b** separation of the two compounds was possible by reversed phase HPLC using reversed phase silica gel (120 Å, 3 μm, C8) as the stationary phase. After complete separation, cleavage of the SEM protecting group with $SnCl_4$ gave the required interstrand crosslink versions of the putative spore photoproducs **8a** and **8b**.

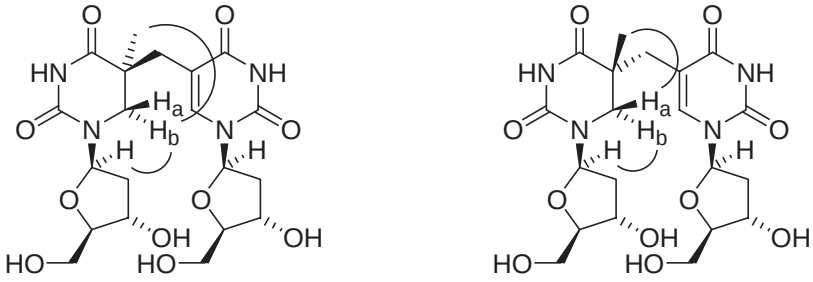


Scheme 2. Synthesis of the interstrand crosslink version of the spore photoproduct. TESCl = Triethylsilylchloride, SEMCl = 2-(Trimethylsilyl)ethoxymethylchloride, DIEA = Diisopropyldiethylamine, TBDMSCl = *tert*-Butyldimethylsilylchloride, NBS = *N*-Bromsuccinimide, DBPO = Dibenzoylperoxide, LDA = Lithiumdiisopropyl-amine, TBAF = Tetrabutylammoniumfluoride.

NOESY experiments were performed to assign the stereochemistry. For the 5*S*-isomer **8a** we measured a strong NOE between H_b and C1'-H. H_b features in addition a strong NOE to the C5 methyl protons. For the 5*R*-isomer **8b**, the measured NOE between H_a and C1'-H is significantly smaller compared to H_b interacting with C1'-H. H_a however features a strong NOE to the C5 methyl protons. The observed strong correlations in the NOE experiment are indicated in Figure 1.

In order to investigate whether compounds **8a** and **8b** would be accepted as a substrate by the enzyme SP-lyase, we have purified the recombinant His-tagged protein from *E. coli* and prepared it in the active holoform containing a (4Fe-4S) cluster under anaerobic conditions. Details of the procedures will be described elsewhere. Standard reaction mixtures contained 50 µM SP-lyase monomer, 1 mM SAM, 3 mM dithionite, and 1 mM of either **8a** or **8b** in 0.1 M Tris HCl buffer, pH 7.0, containing 5 mM DTT together with 0.2 M KCl. Small samples were removed from the assay solution and analysed by reversed phase HPLC (C18 column) for the presence of thymidine, the expected product of the reaction. The chromatograms obtained after 3 h of repair of both isomers (trace 1 and 2) and a control assay containing **8a** but no enzyme (trace 3) are depicted in Figure 1.

a)



8a **8b**

b)

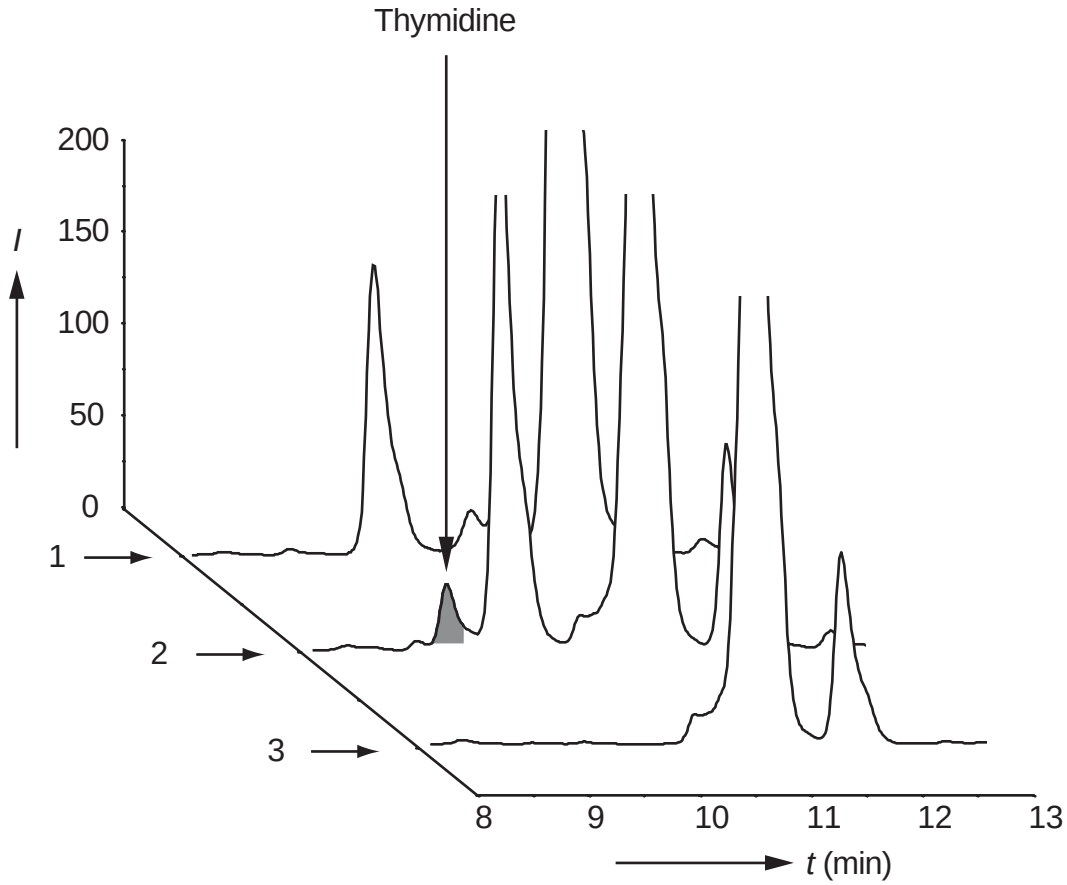


Figure 1. a) Depiction of the two diastereoisomers 5*S* = **8a** and 5*R* = **8b** together with the strong NOE contacts. b) HPLC traces of the enzymatic reaction with **8a** (trace 2) and **8b** (trace 1). The control experiment with **8a** but no enzyme is shown in trace 3. Conditions: 20 min gradient 0 → 28% buffer B (buffer A: 0.1% TFA in water, buffer B: 0.1% TFA in 50% acetonitrile). The additional peaks in the chromatogramm are also

present at the beginning of the reaction and represent other compounds in the reaction mixture.

In all the performed experiments we observe only in the presence of compound **8a** and the enzyme a single new peak in the HPLC experiment with a retention time of 9.67 min. This peak appears consequently only in trace 2. Co-injection of thymidine proofed that this peak is caused by the nucleobase T, which was also confirmed by its UV spectrum. The peak does not form without the enzyme (trace 3) in the control experiment. Most importantly, the new peak does also not form in the presence of the 5*R*-isomer **8b** (trace 1) even after 24 h of incubating **8b** with the enzyme solution. Increasing the concentration of the enzyme and of the substrate increased the efficiency of the repair of **8a** but gave again no detectable thymidine formation in the assay with **8b**. These experiments show for the first time that the 5*R*-isomer is not accepted as a substrate by the enzyme. **8a** is in our hands the only and importantly an efficiently accepted substrate paving the way for detailed enzymatic studies not possible so far.

Additional observation require commentation: During the enzymatic reaction SAM is converted into 5'-deoxyadenosine AdoH. We observed continuous formation of AdoH even in the absence of substrate (data not shown) or in the presence of compound **8b**. Detection of AdoH is therefore not a proper indicator for SP-lyase activity. These data are in agreement with the results reported by W. L. Nicholson[17] but differ from those reported by J. Broderick.[18] Whether the discrepancy is due to the fact that different substrates and now defined substrates, are used in these different studies remains to be established.

Lesion repair is well detectable in our system but overall rather slow. The expected very low binding of the lesion outside the DNA environment and the noted extremely high sensitivity of the enzyme is most likely responsible for this fact. This studies represents the first study in which the enzyme SP-lyase was challenged with defined substrates. The main observation is that the enzyme accepts the interstrand crosslink lesions and only the 5*S*-configured diastereoisomer **8a**. This now raises the interesting questions if the spore photoproduct lesion is indeed the 5*S* crosslink and whether there are distinct enzymes for each diastereosisomer. More experiments are

now needed to further characterize the lesion and the activity of the repair enzyme in order to gain understanding of how nature stores in spores genetic information over thousands of years.

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