The spore photoproduct lyase repairs the 5*S*- and not the 5*R*-configured spore photoproduct DNA lesion†

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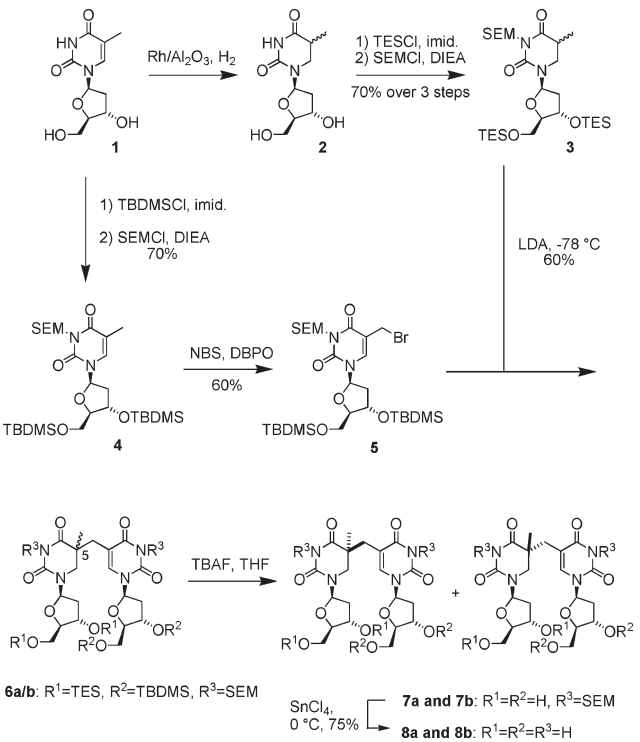
The spore photoproduct lyase is a Fe–S/AdoMet DNA repair enzyme, which directly repairs spore lesions, induced by UV irradiation of spores, using an unknown radical mechanism. The air sensitive radical SAM enzyme was for the first time challenged with synthetically pure substrates. It was found that the enzyme recognizes a synthetic 5*S*-configured spore lesion without the central phosphodiester bond. The 5*R*-configured lesion is in contrast to current belief not a substrate.

Bacteria of the *Bacillus* and *Clostridium* species form metabolically dormant endospores in response to nutrient depletion.[1] Spores are resistant to toxic chemicals, heat, desiccation and can survive over extremely long periods of time.[2] The unusual dehydrated packing of the genetic material inside the spore[3] is responsible for the unusual 50 fold increased resistance to 254 nm UV-light.[4] UV exposure, however, leads to the formation of a unique DNA lesion, the spore photoproduct (SP),[5–7] formed between two adjacent thymidines. This DNA lesion is efficiently repaired in spores by the enzyme SP-lyase.[7–13] The current available data[12,13] suggest that the enzyme belongs to the "radical-SAM" superfamily, which use *S*-adenosyl-L-methionine to generate a 5′-deoxyadenosyl radical (5′-dAdo$^{\bullet}$) as the catalytically active unit which is reduced during the catalytic cycle to 5′-deoxyadenosine (5′-dAdoH).[14–16] The proposed DNA repair mechanism involves H-abstraction from the SP-lesion by 5′-dAdo$^{\bullet}$ followed by a fragmentation of the lesion radical to reform the two thymidines.[12,13,17] The enzyme is remarkable in its ability to precisely control the reactivity of a primary radical in close vicinity to DNA, which is generally readily damaged by free radical chemistry. This in turn must require a highly efficient and selective binding of the lesion in a shielded active site.

In principle, two diastereoisomers of the SP-lesion (5*S* and 5*R*) can form upon UV-irradiation of spore DNA. Since all enzymatic studies were so far only possible using crude UV-irradiated spore DNA as a substrate, it is unclear if both or just one diastereoisomer is formed and repaired.[18]

Further progress in our understanding of the lesion formation and repair process in spores requires the development of synthetic SP-lesions,[19] first as substrates for a SP-lyase assay, and also for structural studies that can clarify the lesion recognition process. Herein we describe the synthesis of a 5*S*- and 5*R*-SP substrate. Our compounds **8a** and **8b** lack the central phosphodiester linkage. Repair of these compounds would generate thymidine which is rapidly detectable by HPLC, which facilitates enzymatic investigations.

The synthesis (Scheme 1) was achieved based on earlier work reported by Begley *et al.*[20,21] The starting point is thymidine **1**, which is first hydrogenated to give dihydrothymidine **2**. Protection of the hydroxyl groups and of the ring imide was performed with



Scheme 1 Synthesis of the interstrand crosslink version of the spore photoproduct lesion. TESCl = triethylsilyl chloride, SEMCl = 2-(trimethylsilyl)ethoxymethyl chloride, DIEA = diisopropylethylamine, TBDMSCl = *tert*-butyldimethylsilyl chloride, NBS = *N*-bromosuccinimide, DBPO = dibenzoylperoxide, LDA = lithium diisopropylamide, TBAF = tetrabutylammonium fluoride.

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† Electronic supplementary information (ESI) available: Synthesis of the SP-isomers **8a** and **8b**, HPLC chromatograms of the separated isomers **7a** and **7b**, ^{1}H-NMR spectra from **8a** and **8b**, analytical data of the bridged SP-isomers **9a** and **9b** and NOE data for the isomers **8a/b** and **9a/b**. See DOI: 10.1039/b514103f

triethylsilyl chloride (TESCl) and 2-(trimethylsilyl)ethoxymethyl chloride (SEMCl) to give compound **3**. A second batch of thymidine was *tert*-butyldimethylsilyl- (TBDMS) and SEM-protected to yield **4** which was 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 methylene linked bis-thymidine compound as two diastereoisomers (**6a** + **6b**). Cleavage of the TES and TBDMS groups with tetrabutylammonium fluoride (TBAF) furnished the SEM-protected diastereoisomers **7a** and **7b**.

7a and **7b** were separated by reversed phase HPLC (120 Å, 3 μm, C8). Final cleavage of the SEM protecting group with $SnCl_4$ furnished the putative SP-lesion monomers **8a** and **8b** (for HPLC chromatograms of the separated diastereomers **7a** and **7b** and the ^{1}H-NMR spectra of isomers **8a** and **8b** see ESI†). For the first time, this modified synthesis allowed us to isolate the spore photoproduct with an open backbone and to deliver substrates for enzymatic studies that are diastereomerically pure and available in sufficient quantities.

Quantitative NOESY experiments allowed us to assign the 5*S* configuration to **8a** and the 5*R* configuration to **8b**. As indicated in Fig. 1, for the 5*R*-isomer **8b** we measured strong NOEs between H_a and the methyl group protons and of the C1′–H with H_b. For **8a** in contrast, we determined strong NOEs between C1′–H, H_b and the methyl protons. This assignment was confirmed with the structurally less flexible derivatives of **9a** and **9b**, in which the central phosphodiester group was replaced by a diester bridge (see ESI†).

In order to investigate whether the pure compounds **8a** and **8b** are accepted as substrates by the SP-lyase enzyme, we purified the recombinant *B. subtilis* protein with a $(His)_6$ tag at the N-terminus to homogeneity according to published procedures[12] and prepared it in the active holoform containing a (4Fe–4S) cluster under strictly anaerobic conditions. Standard anaerobic reaction mixtures contained 50 μM SP-lyase monomer, 1 mM SAM, 3 mM dithionite, and 1 mM of either **8a** or **8b** in 0.3 ml of 0.1 M Tris HCl buffer, pH 7.0, containing 5 mM DTT together with 0.2 M KCl. After 3 h, the reaction was stopped and the assay solution was analysed by reversed-phase HPLC (100 Å, 5 μm, C18 Agilent column). The HPLC gradient was chosen in order to allow separation and hence detection of thymidine in the assay solution, which is the only expected product of the repair reaction (Fig. 1).

We first investigated the formation of thymidine in the presence of either **8a** or **8b** as substrate. To our surprise, we could detect thymidine formation only if the 5*S*-configured compound **8a** was present (Fig. 1). Thymidine formation increased almost linearly during the first three hours (enzyme activity: 0.4 mol (mol enzyme)$^{-1}$ h^{-1}; Fig. 1). Then the reaction stops probably because of enzyme denaturation. Formation of thymidine was confirmed by UV analysis and subsequent co-injection of thymidine. This peak does not form in the control experiment where no enzyme is present (data not shown). In the case of **8b**, neither the addition of further enzyme nor prolonged reaction times (up to 24 h) gave rise to a peak corresponding to thymidine (data not shown). No alternative products deriving from **8b** were detected either. These results show that only **8a** can function as a substrate in the enzyme reaction. As shown by HPLC (Fig. 1 and ESI†), 5′-dAdoH, the product of reductive cleavage of SAM, was also formed during the enzyme reaction. In our experiments the enzyme reaction produces

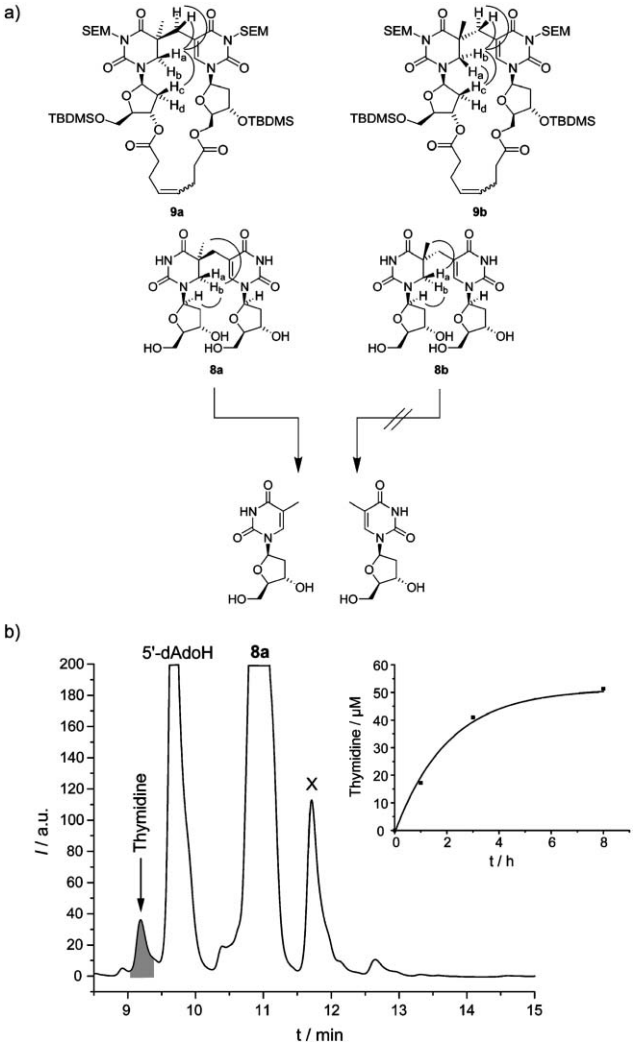

Fig. 1 a) Depiction of the two diastereoisomers 5*S* = **8a** and 5*R* = **8b** and of **9a** and **9b** together with the measured strong NOE contacts. Repair of the lesion by SP-lyase. b) HPLC chromatogram of the enzymatic reaction with **8a**. In the case of **8b** and in the control experiment (**8a** without enzyme) no thymidine could be detected (data not shown). Conditions: 20 min gradient 0 → 28% buffer B (buffer A: 0.1% TFA in water, buffer B: 0.1% TFA in 50% acetonitrile). The peak denoted X is a component derived from the solution containing the reconstituted SP-lyase. The inset shows the time-dependent formation of thymidine during the assay with **8a**.

more 5′-dAdoH than thymidine (Fig. 1 and ESI†), reflecting a decoupling between SAM reduction and thymidine formation. Such a decoupling has been observed in the case of other radical-SAM enzymes. As reported by Rebeil and Nicholson,[12] we showed that SAM can be reductively cleaved by SP-lyase, even in the absence of substrate or in the presence of **8b** (ESI†). On the other hand, the fact that more 5′-dAdoH was formed in the presence of **8a** provides further evidence that indeed only **8a** is able to function as a proper substrate for the repair reaction (ESI†).

In conclusion, this is the first study in which the enzyme SP-lyase was challenged with defined substrates. 5′-dAdoH formation was strongly increased in the presence of **8a**. In addition we observe only in the enzyme mixture containing **8a** formation of

thymidine as the expected "repair" product. Both results provide strong evidence that only the 5*S* configured product is able to function as a substrate for the repair enzyme SP-lyase. The data suggest in contrast to earlier reports[20] that the natural lesion is also 5*S* configured. The results of the present study now pave the way for detailed enzymatic and crystallographic investigations into the SP-lyase lesion recognition and repair process.

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