

Article

Biocatalytic Silylation: The Condensation of Phenols and Alcohols with Triethylsilanol

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Abstract: Silicatein- α (Sil α), a hydrolytic enzyme derived from siliceous marine sponges, is one of the few enzymes in nature capable of catalysing the metathesis of silicon-oxygen bonds. It is therefore of interest as a possible biocatalyst for the synthesis of organosiloxanes. To further investigate the substrate scope of this enzyme, a series of condensation reactions with a variety of phenols and aliphatic alcohols were carried out. In general, it was observed that Sil α demonstrated a preference for phenols, though the conversions were relatively modest in most cases. In the two pairs of chiral alcohols that were investigated, it was found that the enzyme displayed a preference for the silylation of the *S*-enantiomers. Additionally, the enzyme's tolerance to a range of solvents was tested. Sil α had the highest level of substrate conversion in the non-polar solvents *n*-octane and toluene, although the inclusion of up to 20% of 1,4-dioxane was tolerated. These results suggest that Sil α is a potential candidate for directed evolution towards future application as a robust and selective biocatalyst for organosiloxane chemistry.

Keywords: Silicatein; condensation; silyl ether; organosiloxanes; biocatalysis

1. Introduction

During the multi-step chemical synthesis of complex molecules, silyl ethers are often employed for the protection of hydroxyl groups [1-7] where their utility arises from orthogonality to other commonly used acid- and base-labile protecting groups. Typically, the introduction of these silyl groups involves the use of an electrophilic silylating reagent, such as a silyl chloride or triflate (which are themselves produced from the corresponding silyl chloride) [8]. Trimethylsilylation of alcohols has also been demonstrated with *N,O*-bis-silyl trifluoroacetamide [9], *N,N'*-bis-silyl urea [10], and hexamethyldisilazane [11]. It has further been known for many years that the silylation of alcohols can be affected with silanes (silyl hydrides) by dehydrogenative condensation, catalysed by transition metals or strong Lewis acids [12-14]. Alternatively, silylation can also be affected by the activation of the silanol toward attack by an alcohol (or phenol) under Mitsunobu conditions [15].

However, in all cases the necessary reagents are energy intensive to produce, and their use results in the generation of stoichiometric amounts of by-products that are hazardous or environmentally undesirable. In this regard, the capability to silylate hydroxy groups through the condensation of the corresponding silanol and alcohol would circumvent the need for reactive reagents and only release water as the by-product. However, only one example of this dehydrative condensation has been reported, catalysed by rare-earth Lewis acids such as Yb(OTf)₃ and Sc(OTf)₃ [16]. It would be preferable, therefore, to identify a reaction pathway that better conforms to the principles of green chemistry. In

particular, the harnessing of biological catalysts that can be sustainably sourced and avoids any requirement for rare metals [17].

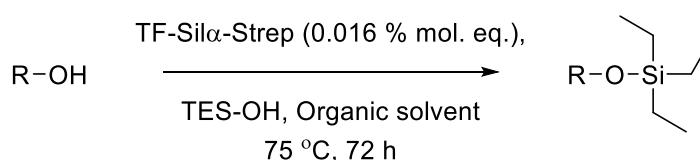
Silicatein- α (Sil α), an enzyme responsible for the condensation of inorganic Si-O bonds in marine demosponges [18,19], has also previously been shown to catalyse the condensation of a variety of organosilanols with aliphatic alcohols to give the corresponding silyl ether [20]. This enzyme is monomeric and does not require any co-factors for activity, and may therefore offer an alternative biocatalytic approach for silylation that is relatively benign. However, the general substrate scope of this enzyme is currently not well established and requires further investigation before synthetically useful methods can be subsequently developed.

In this study, the substrate scope from the perspective of the alcohol component was investigated, by surveying the enzyme's ability to form triethylsilyl ethers with a range of phenols and aliphatic alcohols. In addition, Sil α 's tolerance to a variety of polar and non-polar solvents was tested, to examine the optimum reaction conditions for Sil α in organic media. In the original report of Sil α -catalysed condensations, a preference for phenolic hydroxy groups was shown [20]. However, more recent studies found that the hexahistidine affinity tag used for isolation of the enzyme was also contributing to non-specific catalysis [21]. Thus, in order to more accurately determine the substrate scope and catalysis mediated by the enzyme active site itself, an enzyme construct consisting of the ribosomal chaperone protein trigger factor was fused to the *N*-terminus and a Strep II tag fused to the *C*-terminus was employed in this study.

2. Results and Discussion

2.1. Triethylsilylation of phenols

The triethylsilylation of various substituted phenols was first examined, to investigate the effect of the substituents on this enzyme-catalysed reaction. Chloro, methyl and methoxy substituents were chosen to represent electron donating and withdrawing groups; and all three positions (*ortho*, *meta* and *para*) were investigated. The condensation reactions were carried out based on the procedure previously reported [20], whereby the enzyme was used in the form of a lyophilised solid (in a matrix of potassium salts and 18-crown-6) and the reactions carried out in organic solvent at 75 °C (Scheme 1). The product conversions were measured after 72 h by GC-MS.



Scheme 1. General reaction scheme for the condensation of the alcohol with triethylsilanol (TES-OH) to form the corresponding silyl ether. R = aliphatic or substituted phenyl groups.

For all phenols tested, some degree of product formation was observed even in control reactions where the enzyme was omitted. However, in all cases the inclusion of Sil α resulted in an enhancement in conversions, though some were deemed not to be statistically significant (Figure 1). Unsubstituted phenol was well accepted by the enzyme and gave a gross conversion of 88 % after 72 h and a net conversion of 61 %, after subtraction of the percentage conversion from the non-enzymatic reaction (Table S1 in Supplementary Information). The high net conversions were also found with *p*-methoxyphenol at 62 % (75 % gross). This result equated to a nearly 6-fold higher conversion for the enzyme-catalysed reaction *vs.* the non-enzymatic reaction (i.e. 75 *vs.* 13 %). *o*-Methoxyphenol was the least reactive substrate under these conditions, giving gross and net conversions of 15 and 11 %, respectively. Even so, this result still represented a 3.7-fold increase in conversion attributable to the enzyme since the control reaction gave very little conversion. In all

cases, the remaining material was the unconverted alcohol, silanol and the disiloxane self-condensation product.

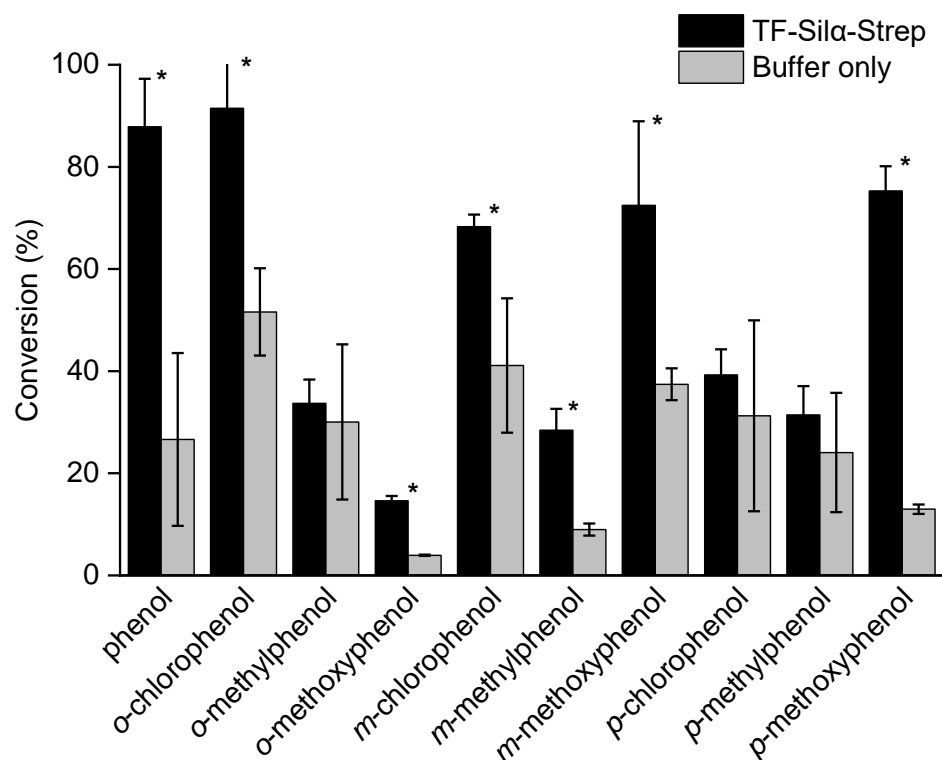


Figure 1. Graph of percentage conversions of phenols to the corresponding silyl ethers after 72 hours. The error bars indicate standard deviations. A one-tailed Student's t-test assuming unequal variance was performed, comparing each enzyme to its control. Comparisons resulting in a $p < 0.05$ were deemed to be significant and marked with *.

In comparing the *ortho*-substituted substrates, 3.7 and 1.8-fold enhancements in the conversion were observed for the methoxy- and chlorophenols, respectively. There was no significant difference in conversion for *o*-methylphenol compared to its control. Since the methoxy-bearing substrate is the most sterically demanding, its preference could be due favourable electronic interactions with the enzyme such as hydrogen bonding or dipole-dipole interactions. Similar interactions could also be responsible for the enhancement observed with *o*-chlorophenol.

The phenols with the *meta*-substituents all exhibited enzymatic enhancements to their conversion that were statistically significant, but no trends could be identified. The greatest degree of enzymatic enhancement was found with *m*-methylphenol with a 3.2-fold improvement over the control, though the net percentage conversion was low at just 19%. The methoxy- and chloro-substituted phenols gave higher absolute conversions, but lower levels of enzymatic enhancement at 1.9 and 1.7-fold, respectively; since the control reactions already gave relatively high conversions even in the absence of the enzyme.

Of the three *para*-substituted phenols, only *p*-methoxyphenol showed a statistically significant conversion enhancement. Indeed, the enzyme gave the highest conversion improvement with this substrate amongst all the tested phenols (Figure 1). Since this substrate presents the bulkiest group of the three *p*-substituted phenols, it is unlikely that this preference is due to steric factors and suggests that the molecule is forming specific interactions that are favourable to either binding or catalysis.

From the perspective of the type of substituent (Figure S1 and Table S1 in Supplementary Information), the chlorophenols generally gave higher conversions for both enzyme catalysed and uncatalysed reactions, and thus the lowest fold improvements (< 1.8

in all cases). Conversely, methoxyphenols gave low conversions in the control reactions but were the most improved by the addition of the enzyme with fold increases ranging from 1.9 – 5.8. The cresols (methylphenols) showed a mixed picture in terms of fold increase but exhibited generally low gross and net conversions.

Overall, no clear trends could be identified either in terms of the type or position of the substituent. An analysis of gross and net conversions, as well as fold increase also showed no clear trends with respect to substrate pK_a or Hammett substituent constants [22]. Thus, the results must arise from a complex interplay of stereoelectronic interactions in the active site of the enzyme. However, as the crystallographic structure of the active enzyme is currently unknown, it is not possible to infer specific interactions.

2.2 Triethylsilylation of aliphatic alcohols

The condensation of a range of alcohols was then investigated under identical reaction conditions. Initially, 1-octanol was tested but gave no conversion above the control reaction after even 192 h (Figure 2, Table S1 in Supplementary Information). This result contrasts with the results from the same protein bearing a hexahistidine affinity tag that gave a net conversion of 86 % after just 72 h [20], and is consistent with the observations from a more recent report showing that the exchange of this tag for a streptavidin affinity tag resulted in a diminishment of activity [21]. Similar results are seen with *E*-3-penten-2-ol, with essentially no activity above baseline.

Two pairs of chiral alcohols, 2-octanol, and 1-phenylethanol, were also investigated in this survey to assess any potential enantioselectivity displayed by the enzyme. Notably, despite no significant conversions with 1-octanol or (racemic) 3-penten-2-ol, both enantiomers of 2-octanol did give significant conversions above that of the control reactions. These conversions are extremely low, at 0.8 and 1.9 % (net) for the *R*- and *S*-enantiomers, respectively; but were statistically significant. Furthermore, these values represented 6.3 and 38.8-fold increases in conversion compared to the controls, respectively. This result thus demonstrates that the enzyme preferentially catalysed the condensation of the *S*-enantiomer. For the phenylethanols, slightly higher gross conversions of 2.2 and 3.2 % (net) were observed, for the *R*- and *S*-enantiomers respectively. However, the 25.5 and 20-fold increases compared to the control indicated a lower level of enantioselectivity.

Only very general trends regarding the reactivity could be drawn from these results. The aliphatic alcohols were overall less reactive than the phenols, in both enzymatic and control reactions, which may be related to their Brønsted acidity. No clear insights were gained when comparing the individual aliphatic alcohols. Indeed, the results superficially appear to be contradictory. Large hydrophobic alkyl chains such as that presented by the 2-octanols are accepted (albeit with low conversion) but 1-octanol is not. Likewise, both 1-phenylethanols are accepted but *rac*-*E*-3-penten-2-ol is not. Substrates containing an aromatic ring appear to give higher conversions (the phenols and phenylethanols) and that suggests preferential binding with the enzyme binding site. This observation appears consistent with earlier computational modelling that indicated a possible cation- π interaction between the ring and a nearby arginine residue [20]. Conversely, the very low level of activity of Sil α with the aliphatic substrates could simply be due to the lack of any attractive interactions with these otherwise largely unfunctionalized molecules.

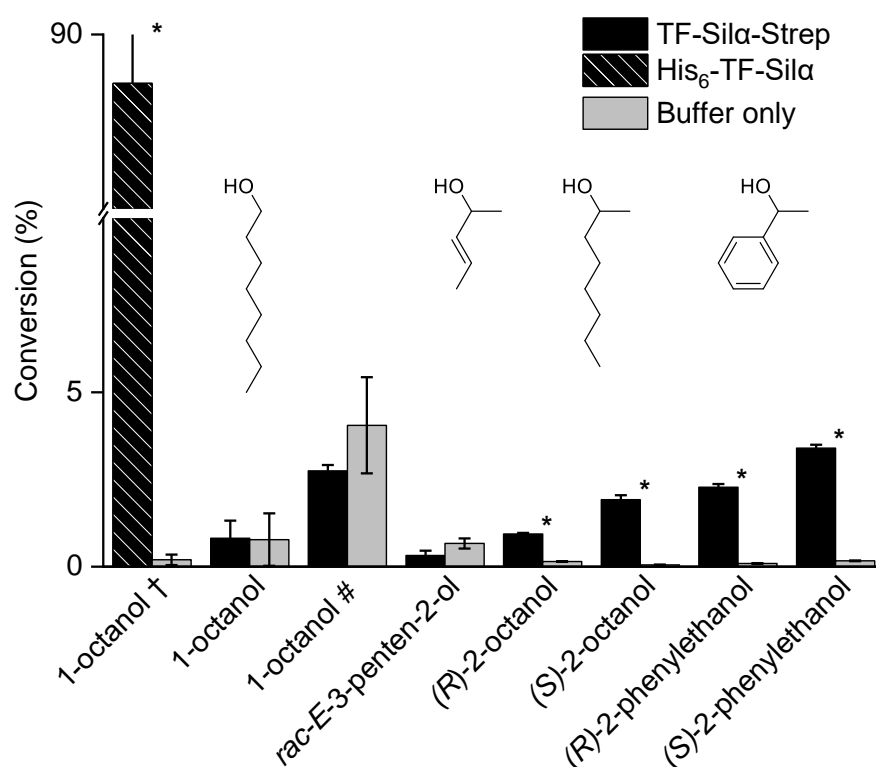


Figure 2. Graph of percentage conversions of alcohols to the corresponding silyl ethers after 72 h. The error bars indicate standard deviations. A one-tailed Student's t-test assuming unequal variance was performed, comparing each enzyme to its control. Comparisons resulting in a $p < 0.05$ were deemed to be significant and marked with *. The † indicates data for the hexahistidine-tagged enzyme taken from ref. 20. The # indicates a reaction time of 192 h. The structures of the alcohols are also shown inset.

2.3 Screening of reaction media.

The current procedure for silyl ether condensations with Silα is based on previous work that utilises octane as the reaction medium [20]. However, this solvent greatly limits the range of substrates that can be applied due to their low solubility. Thus, in an attempt to address this shortcoming, a selection of solvents was tested. Several polar solvents were chosen (ethyl acetate, tetrahydrofuran, 1,4-dioxane, and diisopropyl ether), as well as toluene as a model aromatic solvent. *m*-Methoxyphenol was chosen as the substrate as the above results showed a moderate conversion, allowing for leeway to demonstrate both increases and decreases in conversion. As before, the reactions were carried out using lyophilised enzyme at 75 °C and analysed by GC-MS after 72 hours. In common with the earlier results, some silyl ether product was formed in all cases, even when the enzyme was omitted (Figure 3, Table S2 in Supplementary Information). Increases in conversion above that of the control reactions were observed with the non-polar solvents *n*-octane and toluene, with both giving similar net and gross conversions. In contrast, no net conversion was observed in any of the polar solvents.

To gain further insights, an analysis was performed to correlate net conversion with solvent polarity. For this purpose, the solvent polarity was quantified using empirical “normalised electronic transition energies” (E_T^N) as described by Reichardt [23,24]. This measure is based on the energy of the $\pi \rightarrow \pi^*$ transition of a solvatochromic dye in that solvent and adjusted to a range of between 0.0 (for tetramethylsilane) and 1.0 (water). Notably, this analysis showed that the silanol condensations were sharply demarcated, whereby solvents with $E_T^N > 0.1$ gave essentially no conversions over the control experiments (Figure 3, Table S2 in Supplementary Information).

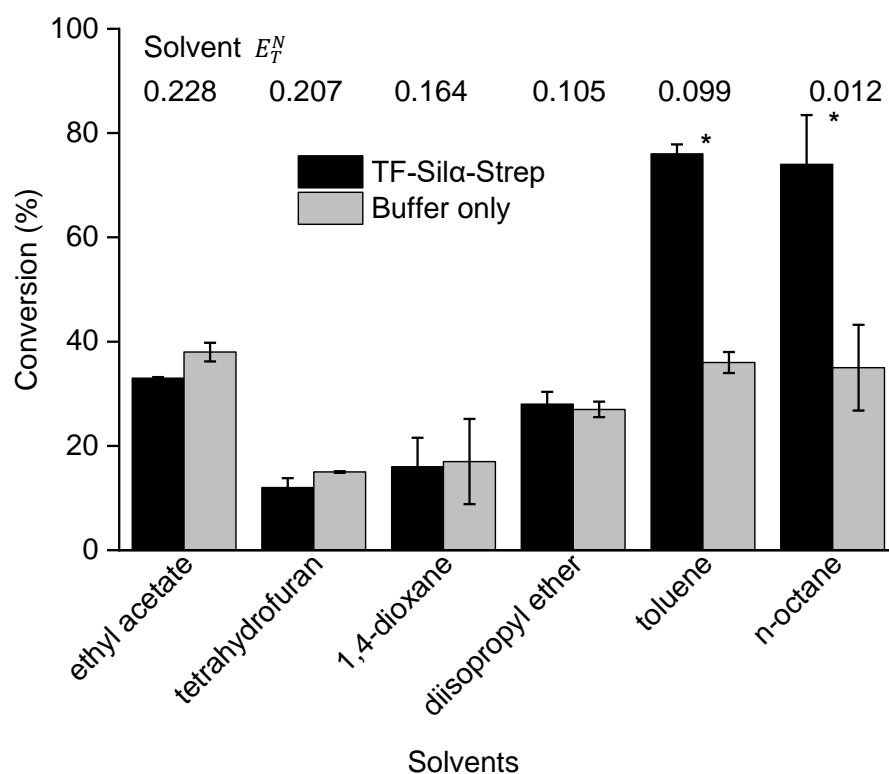


Figure 3. Graph showing percentage conversion of *m*-methoxyphenol and triethylsilanol in a range of solvents after 72 hours. A one-tailed Student's *t*-test assuming unequal variance was performed, comparing each enzyme to its control. Comparisons resulting in a $p < 0.05$ were deemed to be significant and marked with *. The normalised electronic transition energies for the corresponding solvents are shown inset.

It has long been known that when using lyophilised enzymes in organic solvents, non-polar solvents generally give in a higher level of activity, because the more polar (or hydrophilic) solvents remove the essential aqueous monolayer that surrounds the lyophilised enzyme and causes protein denaturation [25,26]. The results presented here conform to this postulation, and therefore suggest that catalysis requires a correctly folded (not denatured) enzyme; i.e. they are not solely due to simple acid-base catalysis afforded by the presence of acidic or basic amino acid residues. Indeed, acid-base catalysis via a classical S_N2 mechanism would have been expected to give higher conversions in polar aprotic solvents, which is not the case here.

As attempts to increase the polarity of the reaction media with a single solvent were unsatisfactory, mixtures containing increasing proportions of 1,4-dioxane in *n*-octane were assessed. 1,4-Dioxane was selected due to its lower polarity (therefore less likely to remove the aqueous monolayer) and boiling point (101 °C) that more closely matched that of *n*-octane. In general, the net conversion fell with increasing proportions of polar solvent (Figure 4, Table S3 in Supplementary Information). Reactions with 30% 1,4-dioxane gave no statistically significant difference between the enzyme and control conversions, presumably due to the removal of structural water crucial to enzyme function, as mentioned above.

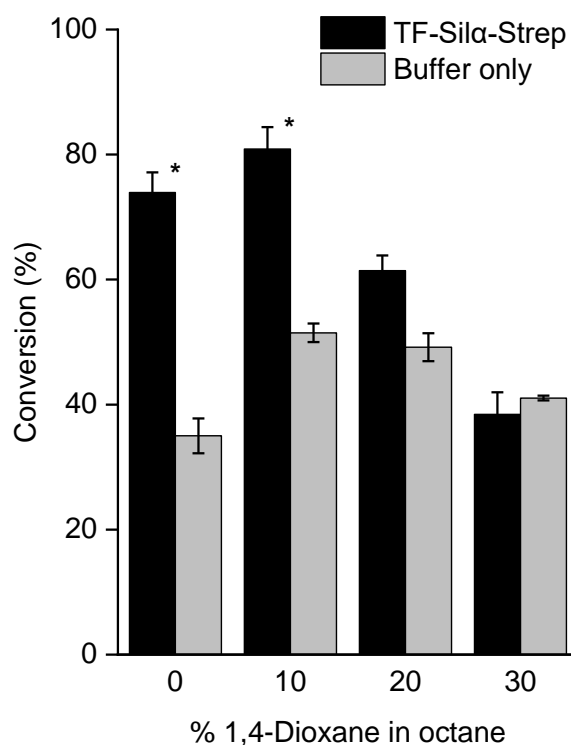


Figure 4. Graph showing percentage conversion of *m*-methoxyphenol and triethylsilanol in varying percentages of 1,4-dioxane in *n*-octane (solvent mixtures) after 72 hours. A one-tailed Student's *t*-test assuming unequal variance was performed, comparing each enzyme to its control. Comparisons resulting in a $p < 0.05$ were deemed to be significant and marked with *.

4. Materials and Methods

4.1 Materials and Equipment

All solvents and reagents were purchased from either Sigma-Aldrich (now Merck), VWR or Fisher Scientific. All solvents used were supplied as anhydrous except *n*-octane and isopropyl ether, and used without further purification. Authentic samples of all product silyl ethers were prepared by conventional synthetic silylation of the corresponding alcohols with chlorotriethylsilane under basic conditions [8] (see Supplementary Information) and were used as standards for the GC-MS analysis. The enzyme was heterologously produced in *E. coli* as previously described [21].

The condensation reactions were carried out in crimp-sealable 8 mm vials (Chromacol C4008-741) that were heated and shaken on an Eppendorf Thermomixer 5350. GC-MS analyses were carried out using Agilent 5975 Series MSD, using the experimental parameters stated below (Table 1).

4.2 Preparation of lyophilised enzyme and matrix

The purified enzyme was buffer exchanged into the lyophilising buffer (100 mM KH_2PO_4 , 100 mM K_2HPO_4 , 20 mM KCl, pH 7) via PD-10 gravity fed desalting columns in batches of 2.5 mL (with multiple columns used in series for large batches). The protein was adjusted to 5 mg mL⁻¹ and 18-crown-6 was added to a 0.04 mM concentration in the final solution. 100 μL aliquots of the enzyme solution were placed in the glass vials, flash frozen by plunging into liquid nitrogen and then lyophilised. For the negative control where the enzyme was omitted, 100 μL aliquots of lyophilising buffer with 18-crown-6 (without the enzyme) were flash frozen and lyophilised.

Table 1. Summary of GC-MS experimental parameters.

Parameter	Setting
Instrument	Agilent 5975 Series MSD
Carrier Gas	9.9995% ultra-high purity helium
GC Inlet, Spin	240 °C, split flow 100 mL min ⁻¹ , split ratio 50
MS Ionisation	Electron ionisation
GC Temperature Program	50 °C (2 min) → 240 °C (8.5 min) at 30 °C min ⁻¹ , 10.7 min total run time
GC Column	VF-5ht
Volume Injected	1 µL

4.3 Enzymatic condensation reactions

A stock solution was first prepared by mixing the desired alcohol (1.26 mmol, 420 mM) and triethylsilanol (6.33 mmol) in the desired solvent (3 mL). 100 µL of this mixture was added into each vial containing the lyophilised enzyme (see above) and the vial crimp sealed. Reaction vessels were then heated at 75 °C while shaking at 650 rpm. At the desired time point, hexane (1 mL) was added, and the mixture centrifuged (17,000 g, 10 minutes) to separate the solid matter. 1 mL of the supernatant was transferred to a clean vial and subjected to GC-MS analysis. Each reaction was performed in triplicate, and error bars presented in the figures refer to the standard deviation of these three independent data point measurements. For quantification of conversion rates, the GC-MS was first calibrated (Figures S2–S13 in Supplementary Information) using the synthetically prepared standards.

5. Conclusions

In summary, a survey into the reactivity of TF-Sil α -Strep with a selection of aromatic and aliphatic alcohols has been conducted. In general, these enzymatic silyl condensations show a preference for aromatic alcohols (i.e. phenols) over aliphatic alcohols, as evidenced by much higher net conversions. However, greater improvements in conversions were achieved with the chiral aliphatic alcohols, as quantified by the fold increase, since the uncatalysed reactions with these alcohols show proportionally much lower levels of product formation. In addition, Sil α has a preference for the *S* enantiomers of the substrates tested, albeit with only low levels of conversion. A subsequent survey of solvents for this reaction showed that condensation could be effected only in non-polar solvents ($E_T^N > 0.1$), though the addition of a small amount of polar solvent (e.g. up to 20% 1,4-dioxane) was tolerated.

As the main aim of this study was to investigate the substrate scope of Sil α , these results are not at present synthetically useful except perhaps in a few cases (e.g. phenol, *p*-methoxyphenol). Nevertheless, this intrinsic activity offers a good starting point for directed evolution [27] into expanding the substrate scope of Sil α . These results thus lay the foundation for future exploitation of Sil α 's chemo- and enantioselectivity towards a "silyl etherase" for practical biocatalysis. Additional work in this direction would benefit from screening a wider variety of aromatic hydroxyl groups, and further elucidation of Sil α 's enantioselective capabilities through screening racemic alcohol mixtures.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Graph of percentage conversions of phenols to the corresponding silyl ethers after 72 hours, Table S1: Percentage conversion, net enzymatic conversion, and conversion enhancement for the condensation of aromatic alcohols and triethylsilanol after 72 h, Table S2: Percentage conversion, net enzymatic conversion, and conversion enhancement for the condensation of *m*-methoxyphenol and triethylsilanol in various solvents after 72 h, Table S3: Percentage conversion, net enzymatic conversion, and conversion enhancement for the condensation of *m*-methoxyphenol and triethylsilanol in various mixtures of 1,4-dioxane and *n*-octane, Figure S2 – S13: Calibration graphs of area under the peak corresponding to silyl ethers in the GCMS trace against concentration, compound characterisation data for the authentic silyl ether products.

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