Enzyme Activation by Water-Mimicking Dual-Functionalized Ionic Liquids

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Abstract

Biocatalytic synthesis represents a green approach alternative to metal-catalyzed reactions. However, enzymes typically display much lower catalytic activities in nonaqueous solvents than in aqueous media. To mimic the aqueous environment for enzyme activation, this study designed a series of sixteen dual-functionalized ionic liquids (ILs) that comprising both glycol ether (hydrogen-bond acceptor) and *tert*-alcohol (hydrogen-bond donor) groups. These "water-like" ILs enabled high transesterification activities for immobilized *Candida antarctica* lipase B (CALB) known as Novozym 435 and immobilized *Bacillus licheniformis* protease (known as subtilisin A) respectively. Several water-mimicking ILs containing 2–3 v% water significantly increased the CALB activity by 1.8-fold of that in *tert*-butanol, and 1.6-fold of that in diisopropyl ether (both organic solvents are highly enzyme-compatible). To a smaller degree, subtilisin was activated by these ionic solvents up to 1.2-fold (with 100% selectivity at 2 v% water) than by diisopropyl ether. Fluorescence emission spectra suggested that the characteristic emission maximum peaks were maintained in "water-like" ILs in most cases.

Keywords: biocatalysis, enzyme catalysis, hydrolase, ionic liquid, transesterification

1. Introduction

Enzymes not only act as biocatalysts for numerous biological conversions in nature, but also play a key role in many organic reactions as "green" catalysts. In particular, nonaqueous biocatalysis offer unique advantages over aqueous reactions such as the solubilization of water-insoluble substrates, improved thermal stability of enzymes, enhanced enzyme selectivity, shifting reaction equilibria (e.g., from hydrolysis to synthesis), and minimizing microbial contamination, etc. [1-3]. However, enzyme activity in nonaqueous solvents is highly depressed than in aqueous environment usually by two to five orders of magnitude [1, 4]. A classic example is that proteases (i.e. α-chymotrypsin and subtilisin) were 10^4–10^5-fold less active in octane than in water [1]. Enzyme activity depression in organic solvents can be explained by a number of intrinsic factors including those that can be tackled by solvent engineering, such as limited conformational mobility of enzymes, reduced molecular dynamics of proteins in organic media, and transition state stabilization (i.e. polar transition state is stabilized by water molecules much better than organic solvents) [4-6].

To improve the performance of enzymes in nonaqueous media, conventional solvent engineering identified solvents with suitable polarity and hydrophobicity (in terms of the log *P* scale) leading to an optimized enzyme activity [7, 8]. Additionally, several hydrophilic solvents were recognized as "water-mimicking" or "molecular lubricant" solvents including formamide, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), glycerol, ethylene glycol, and ethylene glycol dimethyl ether although the enzyme activation by these solvent was very limited [9-14]. Furthermore, nonaqueous media for biocatalysis extended from conventional organic solvents to supercritical fluids [15, 16], fluorous solvents [15, 17], gas phase [18, 19], ionic liquids (ILs) [20-23], and deep eutectic solvents (DES) [24-26]. Particularly, due to their structural versality, ILs

have been modified primarily through ether- or hydroxy-functionalization to increase their compatibility with enzymes [6, 27]. A simple reason for incorporating these functional groups is due to the enzyme-stabilizing nature of some hydroxy- or ether-containing molecules such as polyols (e.g. sorbitol and trehalose) and sugars [28, 29], *tert*-butanol [30-36], and ethers (e.g. diisopropyl ether, 2,2-dimethoxypropane and 2-ethoxyethyl ether) [37-41]. Following this rationale, our group recently designed lipase-compatible "water-like" imidazolium and ammonium ILs functionalized with *tert*-alcohol- and short-chain-ether groups, resulting in considerably increased transesterification activities than *tert*-butanol and conventional ILs [42, 43]. These IL structures are called "water-like" because they mimic the water structure by having both ether group (hydrogen-bond acceptor) and *tert*-alcohol group (hydrogen-bond donor). However, these earlier studies only examined isolated structures of imidazolium and ammonium-type ILs, and have not systematically evaluated a series of IL structures (e.g., various glycol chain length) to establish an IL structure–enzyme activity relationship. Thus, the present study aims to synthesize a full range of water-mimicking ILs that carry glycol ether chains with varied length, multiple ether chains, or trimethylsilyl groups in addition to *tert*-butanol group, and to determine their physiochemical properties and compatibility with immobilized *Candida antarctica* lipase B (CALB) known as Novozym 435 and immobilized *Bacillus licheniformis* protease (known as subtilisin A). Based on the IL structure–enzyme activity relationship, this study provides a rational strategy for designing a new class of water-mimicking and enzyme-activating nonaqueous solvents.

2. Experimental

2.1. Materials

Novozym 435 (*Candida antarctica* lipase B (CALB) immobilized on acrylic resin) (Catalog # L4777, Lot # SLBW1544, enzyme activity 11,900 propyl laurate units (PLU)/g), lipase B *Candida antarctica*, recombinant from *Aspergillus oryzae* (Catalog # 62288, Lot # BCBW4983,

powder, beige, 7.9 U/mg), protease from *Bacillus licheniformis* (known as Subtilisin A, Catalog# P5380, Type VIII, lyophilized powder, 7-15 units/mg solid), glutaraldehyde solution (Grade I, 50% in water), *N*-Acetyl-L-phenylalanine ethyl ester (≥98%), *N*-Acetyl-L-phenylalanine (≥99 %), and Bradford Reagent (for 0.1-1.4 mg/ml protein) were acquired from Sigma-Aldrich (St. Louis, MO). Diethylene glycol monomethyl ether (≥99.6%), triethylene glycol monomethyl ether (≥97.0% GC), dipropylene glycol monomethyl ether (mixture of isomers) (> 98.0% GC), tripropylene glycol monomethyl ether (mixture of isomers) (> 93.0% GC), bis(2-ethoxyethyl)amine (≥98.0%), *N*,*N*-bis(2-isopropoxyethyl)amine (≥97.0% by GC, titration analysis), 1-(2-bromoethoxy)-2-(2-methoxyethoxy)ethane (≥94.0%), 1,2-epoxy-2-methylpropane (known as isobutylene oxide; also referred as 1,1-dimethyloxirane, or 2,2-dimethyloxirane), and ethyl sorbate (≥98.0%) were purchased from TCI America (Portland, OR). Polyethylene glycol monomethyl ether with M.W. ~350 (PEG-350) and ethylamine (≥70 wt% in water) was obtained from BeanTown Chemical (Hudson, NH). Lithium bis(trifluoromethylsulfonyl)imide supplied by VWR (Radnor, PA) is the product of Biosynth International Inc. (Oakbrook Terrace, IL).

2.2. Synthesis of glycol- and *tert*-alcohol-dual-functionalized ILs

The first step was glycol sulfonation following modified literature methods [44-47]. Glycol monomethyl ether (~50 g) was mixed with 50 mL THF and 1.7 molar eq. NaOH (dissolved in the same amount of water) to form biphasic layers, followed by the dropwise addition of 1.1 molar eq. benzenesulfonyl chloride, forming white precipitate (salts). The reaction mixture was cooled in ice bath when needed to keep the temperature below 50 °C. When the mixture began to cool down, a gentle heat was applied to maintain the reaction at 40 °C for 5 h under stirring. At the completion of reaction, the mixture was cooled to room temperature and then in ice bath. The white precipitate was filtered off, and THF was removed under vacuum from the filtrate. The slightly yellow liquid

was dissolved in 100 mL CH_2Cl_2 and extracted by 1.0 N HCl twice (100 mL each), followed by further washing with saturated $NaHCO_3$ solution (100 mL) and deionized water (100 mL). The organic layer was dried by Na_2SO_4, followed by filtering off drying salt and evaporating the solvent, producing colorless liquid.

The second step was nucleophilic substitution of glycol sulfonate with monoethylamine [47-49]. Monoethylamine (70 wt% in water, 5 molar eq.) was mixed with 2.0 molar eq. K_2CO_3, followed by the addition of glycol benzenesulfonate (~40 g) in 50 mL THF. The mixture was refluxed for 24 h under stirring. At the end of reaction and after cooling the reaction mixture in ice bath, precipitate was removed through filtration, followed by vacuum evaporation of THF. The biphasic mixture was extracted by 100 mL CH_2Cl_2 (the organic layer was at the bottom in most cases but could be on the top such as in the case of benezenesulfonate of triethylene glycol monomethyl ether), and the organic layer was further washed by brine twice (100 mL each). The organic layer was dried by Na_2SO_4, and slightly brown liquid product was obtained after removing the salt and evaporating the solvent.

The third step was to graft *tert*-alcohol group onto glycol-substituted ethylamine following a modified literature method [42, 50]. Glycol-substituted ethylamine (~30 g, 1.0 molar eq.) was mixed with 100 mL ethanol and sodium acetate (1.1 molar eq.), followed by the addition of 1.2 molar eq. 1,1-dimethyloxirane (i.e. isobutylene oxide). The reaction mixture was refluxed for 24 h under stirring. After cooling the reaction to room temperature then in ice-bath, the solid was filtered off, and ethanol was evaporated under vacuum. After removing ethanol, more solid (sodium acetate) precipitated, which was filtered again with the washing by CH_2Cl_2. The solvent was further evaporated under vacuum to afford a slightly yellow and viscous liquid.

The fourth step was to produce quaternary ammonium salt through alkylation. *tert*-Alcohol- and glycol-grafted tertiary amine (~20 g, 1.0 molar eq.) was dissolved in 100 mL anhydrous acetone, followed by the addition of 1.1 molar eq. methyl iodide. The mixture was wrapped by aluminum foil (to minimize light-initiated reactions) and refluxed for 24 h under stirring. After the solvent was evaporated under vacuum at the end of reaction, the iodide salt was rinsed by diethyl ether twice (50 mL each time) to remove residual reactants and impurities. Trace amount of ether was removed under vacuum. The iodide salt was weighed and dissolved in deionized water; the solution was decolored by activated carbon for 24 h. After filtering off activated carbon, the aqueous solution was used in the last step of reaction.

The final step was to replace iodide with Tf_2N^- anion via anion exchange. Dual-functionalized-ammonium iodide (~20 g, 1.0 molar eq.) was mixed with 50 mL deionized water, followed by a dropwise addition of lithium bis(trifluoromethylsulfonyl)imide (Li[Tf_2N], 1.05 molar eq.) dissolved in 30 mL water. The reaction was stirred at room temperature for 30 min under gentle stirring. After a complete phase separation, the bottom IL layer was dissolved in 100 mL CH_2Cl_2 and extracted by deionized water twice (100 mL each) using a separatory funnel (or until no iodide can be detected in the aqueous layer by $AgNO_3$ test). The organic layer was dried by Na_2SO_4, followed by decantation and evaporation of CH_2Cl_2 under vacuum. The IL product was rinsed by *n*-heptane twice (50 mL each). Trace *n*-heptane was removed under vacuum. The final product was dried in a vacuum oven (80 °C and 25 mmHg vacuum) for one week. IL structures were characterized by ^1H and ^{13}C NMR, ESI-MS, FT-IR and TGA analysis (see *Supplementary Information*).

2.3. Fluorescence emission of free enzymes in ILs

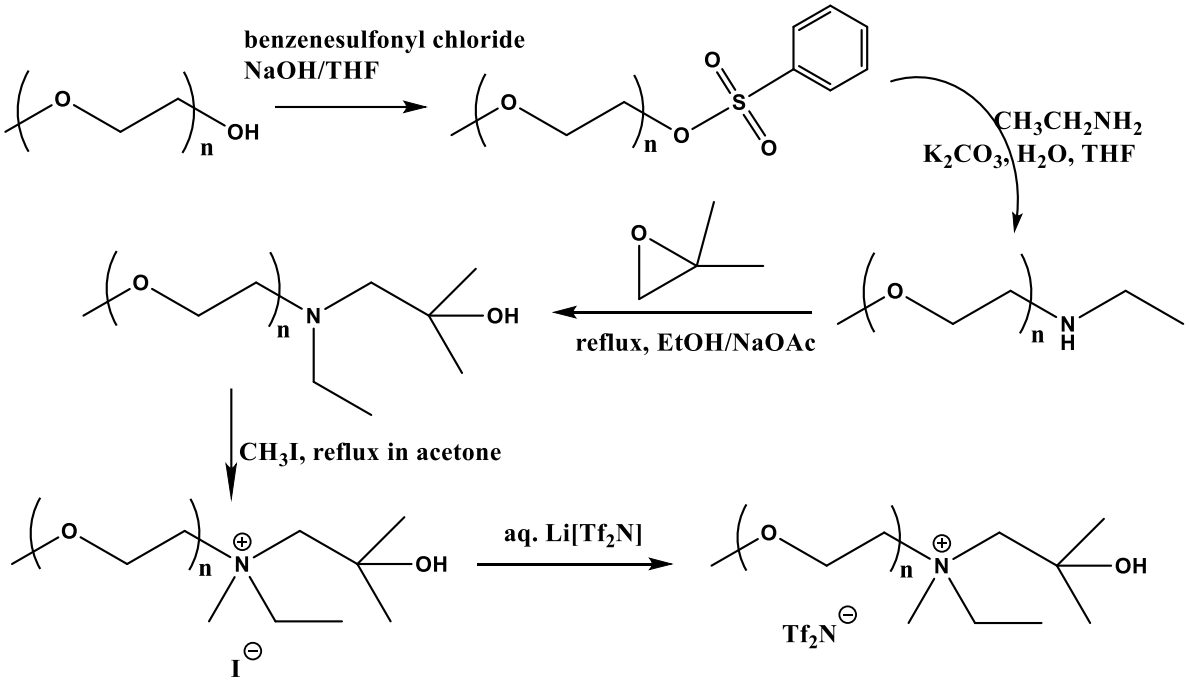
Fluorescence emission spectra of free enzyme (CALB or subtilisin) in different solvents were determined using a Hitachi F-2500 fluorescence spectrophotometer. The enzyme solution was excited at 280 nm to monitor the emission signal of tyrosine and tryptophan residues at ~300 nm. Free enzyme was dissolved in pH 7.5, 20 mM phosphate buffer to prepare 20 mg/mL stock solution. An aliquot of the enzyme solution (20 μL) was mixed with 1.0 mL of solvent (phosphate buffer or IL) in a microcentrifuge tube by turning the tube upside down several times to dissolve or disperse aqueous enzyme in the solvent. The overall enzyme concentration was 0.4 mg/mL. The enzyme mixture was pipetted into a quartz cuvette (1.0 mL volume). Fluorescence measurements were taken at room temperature (~22 °C). The excitation monochromator slit was maintained at 5 nm, and the emission monochromator slit width was also kept at 5 nm. The PMT voltage was set at 400 V and the response time was 0.08 s. Each spectrum was scanned from 250–400 nm at a rate of 300 nm/min. The emission spectrum of each solvent alone was scanned under the same condition, which was deducted from the enzyme emission spectrum in the same solvent.

3. Results and discussion

3.1. Synthesis and characterization of water-mimicking functionalized ILs

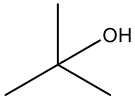
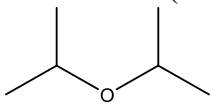
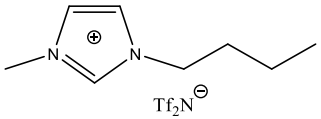
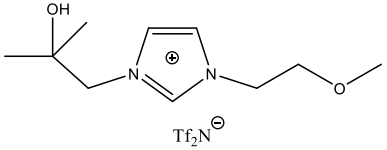
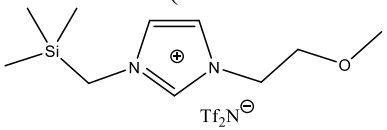
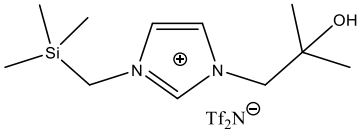
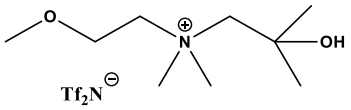
Our recent study described the challenges of synthesizing ILs dual-functionalized with both *tert*-butanol and ether groups (see Scheme S1 in *Supporting Information* of Ref [43]). The present study further expanded previous three-step strategy to a complete five-step route with glycol monomethyl ether as the starting material (Scheme 1). These five steps include the sulfonation of glycol monomethyl ether, nucleophilic attack of sulfonate ester by monoethylamine to yield glycol-functionalized secondary amine, nucleophilic ring-opening of isobutylene oxide by glycol-functionalized amine, quaternization of functionalized tertiary amine by methyl iodide, and an anion-exchange process to produce “water-like” hydrophobic ILs with Tf_2N^- anion. In total, we

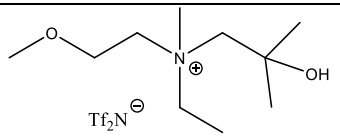
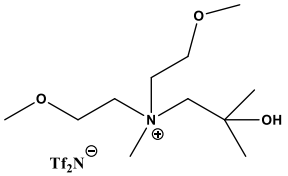
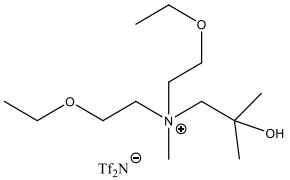
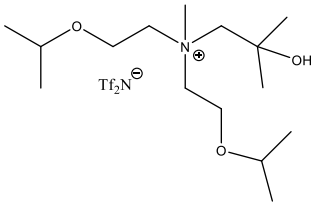
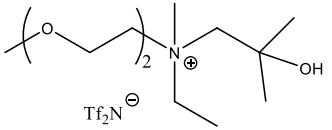
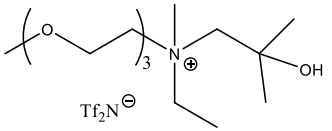
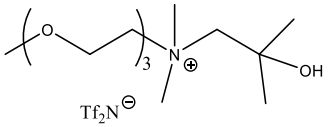
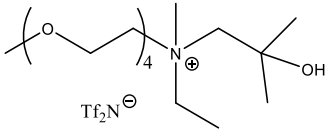
synthesized 16 water-mimicking functionalized ILs (**2**-**17** in Table 1) including several from our recent studies [42, 43] following Scheme 1, and Schemes S1–S8 and S11 in *Supplementary Information*. These water-mimicking ILs cover a number of structural variations (see Table 1): imidazoliums (**2**, **3**, **4**, and **15**) and ammoniums (**6**–**14**, **16**, and **17**); functionalization with ethylene glycol ether (**2**, **3**, **5** and **6**), diethylene glycol ether (**10**), triethylene glycol ether (**11** and **12**), tetraethylene glycol ether (**13**), polyethylene glycol ether 350 (**14** and **15**), dipropylene glycol ether (**16**), tripropylene glycol ether (**17**), trimethylsilyl (**3**, **4** and **15**), and two glycol ether groups (**7**–**9**).

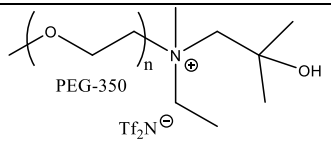
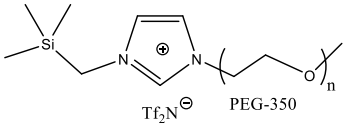
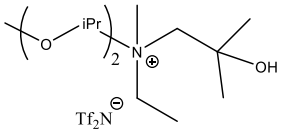
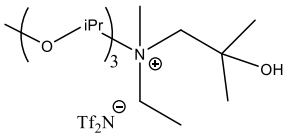


Scheme 1 Five-step strategy to synthesize dual-functionalized ammonium ILs.

Table 1 Dual-functionalized ionic liquids, their physiochemical properties and lipase activity

Solvent (0.02 wt% water) [a]	Dynamic viscosity at 30 °C (mPa s) [b]	Kinematic viscosity at 30 °C (mm^2 s^{-1}) [b]	Density at 30 °C (g cm^{-3}) [b]	T_{der} (°C) [c]	T_{dcp} (°C) [c]	Lipase activity (μmol min^{-1} g^{-1} free CALB) and selectivity [d]
 (0.013 wt% water)	4.31 (25 °C)[51]	–	0.7887 (20 °C)[51]	–	–	6.66 (97%)
	0.299 [52]	0.419 [52]	0.7131 [52]	–	–	7.83 (>99%)
1  (0.01 wt% water)	41.4 [53]	28.9 [53]	1.430 [53]	464.0 [53]	406.0 [53]	5.52 (>99%)
2  (0.024 wt% water)	303.03	213.33	1.4205	387.0	329.8	10.24 (>99%)
3 	69.62	51.749	1.3453	430.1	351.2	7.14 (>99%)
4 	437.29	322.13	1.3575	397.4	321.9	9.05 (>99%)
5 	129.27	92.114	1.4034	319.8	303.5	9.85 (>99%)

6		175.31	126.65	1.3841	316.8	297.8	7.37 [43] (>99%)
7		190.42	138.17	1.3782	302.4	286.3	6.73 [43] (>99%)
8		118.51	92.486	1.2813	304.2	289.2	8.98 (>99%)
9		197.43	155.75	1.2676	273.7	269.3	7.23 (>99%)
10		146.58	108.22	1.3545	317.6	302.6	8.61 (87%)
11		123.17	93.356	1.3194	317.2	294.3	9.90 (>99%)
12		84.92	63.12	1.3453	324.1	287.7	6.64 (>99%)
13		152.96	116.29	1.3153	314.5	297.9	8.93 (80%)

14		238.97	183.96	1.2991	307.3	286.1	7.15 (>99%)
15		190.32	149.52	1.2729	392.0	336.4	6.13 (>99%)
16		154.09	121.62	1.2671	361.2	179.4	8.88 (>99%)
17		158.56	126.13	1.2571	348.8	245.7	8.28 (>99%)

Note: [a] A coulometric Karl Fischer titrator was used to measure the water content at 22 °C with Hydranal® Coulomat AG as the analyte. [b] An Anton Paar SVM 3000 viscometer was used to determine the dynamic/kinematic viscosity and density data at 30 °C (except noted otherwise). [c] T_{der} is determined from the maximum in the first-derivative profile of the TGA scan. T_{dcp} is the decomposition temperature measured as the onset of decomposition, using the common criterion of 10% total mass loss. Uncertainties in the temperatures are estimated to be on the order of ±2–3 °C. [d] The transesterification was conducted by adding ethyl sorbate (5 mM) and 1-propanol (0.67 M) in 1.0 mL solvent with the presence of 20 mg Novozym 435 (~ 4 mg free CALB) at 50 °C. The enzyme activity was calculated based on ~4 mg free CALB in 20 mg Novozym 435. All lipase activities were within the margin of error of 5%.

Solvents with low viscosities are desirable reaction media to minimize mass transport limitation and increase the process operability. As a general rule in IL design, the incorporation of an ether group decreases the viscosity while appending an alcohol group increases the viscosity (due to hydrogen bonding) (e.g., IL **3** with dynamic viscosity of 69.62 mPa s *vs* IL **4** with 437.29 mPa s at 30 °C in Table 1) [27]. If both groups are grafted to an IL, a lower viscosity can be achieved by balancing the size of ether chain with other groups to produce an asymmetric cation. Ammonium-based ILs tend to have lower viscosities than imidazolum analogue (e.g., IL **5** with 129.27 mPa s *vs* IL **2** with 303.03 mPa s). Two-ether-chain-functionalization does not appear to significantly influence their viscosities (**7–9** vs **5**). A longer glycol ether chain (up to triethylene glycol ether, see ILs **6**, **10**, and **11**) leads to lower viscosities (up to 123.17 mPa s for IL **11**), but a further elongation of glycol ether chain results in a higher viscosity (ILs **13** and **14**). A shorted alkyl chain (methyl *vs* ethyl) leads to a reduction in IL viscosity (e.g., IL **5** with 129.27 mPa s *vs* IL **6** with 175.31 mPa s; IL **12** with 84.92 mPa s *vs* IL **11** with 123.17 mPa s). It is also known that alkylsilyl or alkylsiloxy functionalization of ILs leads to a considerably lower viscosity (due to weaker ion interactions and flexible Si–O–Si chain) [54, 55] and larger free volume (due to the bulky structure) [56]. Our trimethylsilyl-grafted ILs have considerably lower viscosities than *tert*-butanol-substituted analogues (e.g., IL **3** with 69.62 mPa s *vs* IL **2** with 303.03 mPa s; IL **15** with 190.32 mPa s *vs* IL **14** with 238.97 mPa s).

Short-term thermal stability of ILs was quantified by the TGA analysis using two parameters: T_{der} (the maximum in the first-derivative profile of the TGA scan) and T_{dcp} (the decomposition temperature measured as the onset of decomposition, based on the common criterion of 10% total mass loss) as shown in Table 1 (also in Table S1 and Figures S1–S16 in *Supplementary Information*). [BMIM][Tf_2N] (**1**) has a high thermal stability (T_{dcp} = 406.0 °C),

which is used as a reference point. Water-mimicking ILs have lower thermal stability (most T_{dcp} in the range of 270–330 °C) than alkyl-substituted IL **1**; imidazolium-type ILs (**2**, **3**, **4**, and **15**) are more thermally stable than ammonium-based analogues. A longer ethylene glycol ether chain has a minimum impact on the thermal stability of ILs (comparing **6**, **10**, **11**, **13**, and **14**), but propylene glycol ether chains (ILs **16** and **17** with T_{dcp} of 179.4 and 245.7 °C respectively) seem less stable than ethylene glycol ether chains. In addition, trimethylsilyl chain is more thermally stable than *tert*-alcohol group (T_{dcp}: IL **3** with 351.2 °C *vs* IL **2** with 329.8 °C; IL **15** with 336.4 °C *vs* IL **14** with 286.1 °C). In summary, since most enzymatic reactions are conducted below 100 °C, our water-mimicking ILs are low-viscosity fluids and are thermally stable media under biocatalytic conditions.

3.2. Effect of IL structures on lipase activity

To compare the enzyme compatibility of these "water-like" ILs with conventional solvents, we firstly examined Novozym 435 (immobilized CALB) through a transesterification reaction between ethyl sorbate and 1-propanol as a model reaction (see procedures in Section 3 of *Supplementary Information*) [42, 57]. Table 1 shows the lipase activity in each solvent at a low water content (mostly 0.02 wt%); reaction selectivity (transesterification *vs* hydrolysis) at such a low water environment was mostly >99% (except 97% in *tert*-butanol, and 80% in IL **13**). As a point of reference, three well-known lipase-compatible solvents (i.e., *tert*-butanol [30-36], diisopropyl ether [37-40], and [BMIM][Tf_2N] [58, 59]) produced high transesterification activities (6.66, 7.86, and 5.52 μmol min^{-1} g^{-1} free CALB, respectively in Table 1), which coincide with literature findings that lipase-compatible ILs typically yielded similar enzyme activities as *tert*-butanol but lower activities than diisopropyl ether [6, 20, 21, 23]. In one case, Itoh and co-workers [60] carried out the enzymatic transesterification of (*E*)-4-phenylbut-3-en-2-ol with vinyl acetate

in different solvents, and observed comparable reaction rates in diisopropyl ether and an ether-functionalized IL [$CH_3OCH_2CH_2$-Bu_3P][Tf_2N] using Novozym 435, but a higher reaction rate (1.1-fold increase) in [$CH_3OCH_2CH_2$-Bu_3P][Tf_2N] than in diisopropyl ether using lipase PS coated with an imidazolium alkyl-PEG sulfate IL.

Most of our water-mimicking ILs (containing 0.02 wt% water) in Table 1 afforded higher transesterification activities than *tert*-butanol (except **12** and **15**), and a few of them even produced higher activities than diisopropyl ether (**2**, **4**, **5**, **8**, **10**, **11**, **13**, **16**, and **17**). The highest activities were observed in ILs **2** and **11** were 10.24 and 9.90 μmol min^{-1} g^{-1} free CALB respectively, representing 1.5-fold lipase activation than *tert*-butanol and 1.3-fold activation than diisopropyl ether under a low water content (0.02 wt%). The effect of glycol-chain-length on ammonium-based ILs can be demonstrated by comparing lipase activities in ILs **6**, **10**, **11**, **13**, and **14**; it appears that enzyme activity increased with a longer glycol chain (up to triethylene glycol ether) and then decreased with a longer glycol ether chain. Our recent study [42] pointed out that a longer glycol chain on imidazolium cations resulted in lower lipase activities. Therefore, the cation core and the overall IL structure are essential to their compatibility with enzymes. The grafting of propylene glycol ethers (ILs **16** and **17**) exhibited comparable activities with their ethylene glycol ether analogues (ILs **10** and **11**, respectively). When incorporating two glycol ether chains to IL cations (**7**, **8**, and **9**), the ether chain capped with an ethyl end group (IL **8**) led to a higher lipase activity (8.98 μmol min^{-1} g^{-1} free CALB) than those capped with methyl- (IL **7**) and isopropyl- (IL **9**) groups. The substitutions of *tert*-alcohol or ether group by trimethylsilyl chain [ILs (**3** and **4)** vs **2**, and **15** vs **14** in Table 1] suggests lower lipase activities in all cases. In summary, based on current and previous data [42, 43], dual-functionalized ILs consisting of *tert*-alcohol and suitable-length-glycol ether groups boosted the transesterification activity of immobilized CALB considerably

when compared with organic solvents and conventional ILs. It is rationalized that these IL structures are "water-like" because they possess both ether group (hydrogen-bond acceptor) and *tert*-alcohol group (hydrogen-bond donor), affording water-mimicking environment for the enzyme.

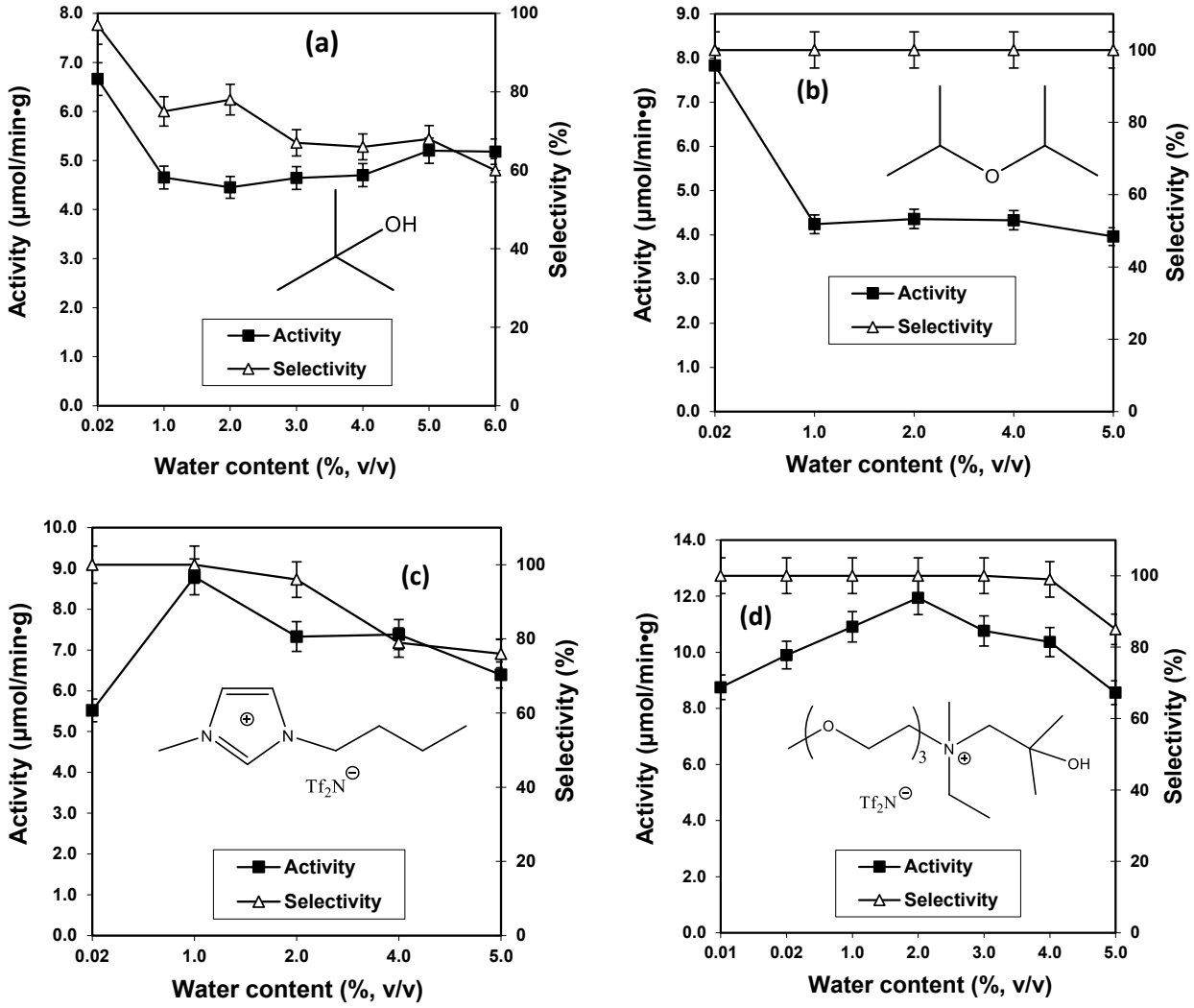
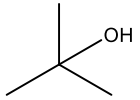
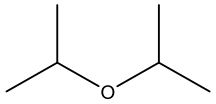
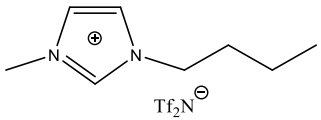
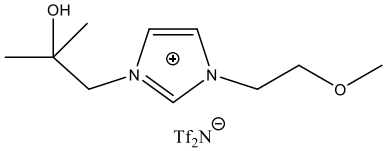
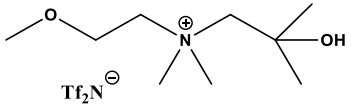
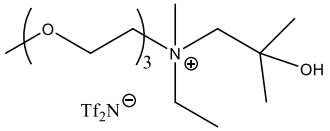
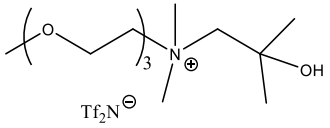


Figure 1 Effect of water content on Novozym 435 transesterification activity in different solvents: (a) *tert*-butanol, (b) diisopropyl ether, (c) [BMIM][Tf_2N] (**1**), and (d) IL **11**.

Table 2 Optimum water contents for highest transesterification activities

Solvent		Novozym 435			Subtilisin		
		Water (v%)	**Lipase activity** [a]	**Lipase selectivity**	**Water (v%)**	**Protease activity** [b]	**Protease selectivity**
		0.017	6.66	97%	5.0	12.65	25
		0.02	7.83	>99%	1.0 2.0	11.76 44.47	100 41
1		1.0	8.80	>99%	2.0 10.0	1.31 10.45	97 20
2		2.0	11.41	>99%	2.0 10.0	3.52 21.79	21 12
5		2.0	11.37	>99%	2.0 10.0	7.04 34.44	18 16
11		2.0	**11.94**	**>99%**	2.0 10.0	**14.28** **55.55**	**100** **28**
12		3.0	**12.21**	**>99%**	2.0 10.0	9.77 24.62	100 20

Note: [a] Lipase activity in the unit of μmol min^{-1} g^{-1} free CALB; [b] Protease activity in the unit of μmol min^{-1} g^{-1} free protease. All lipase and protease activities were within the margin of error of 5%.

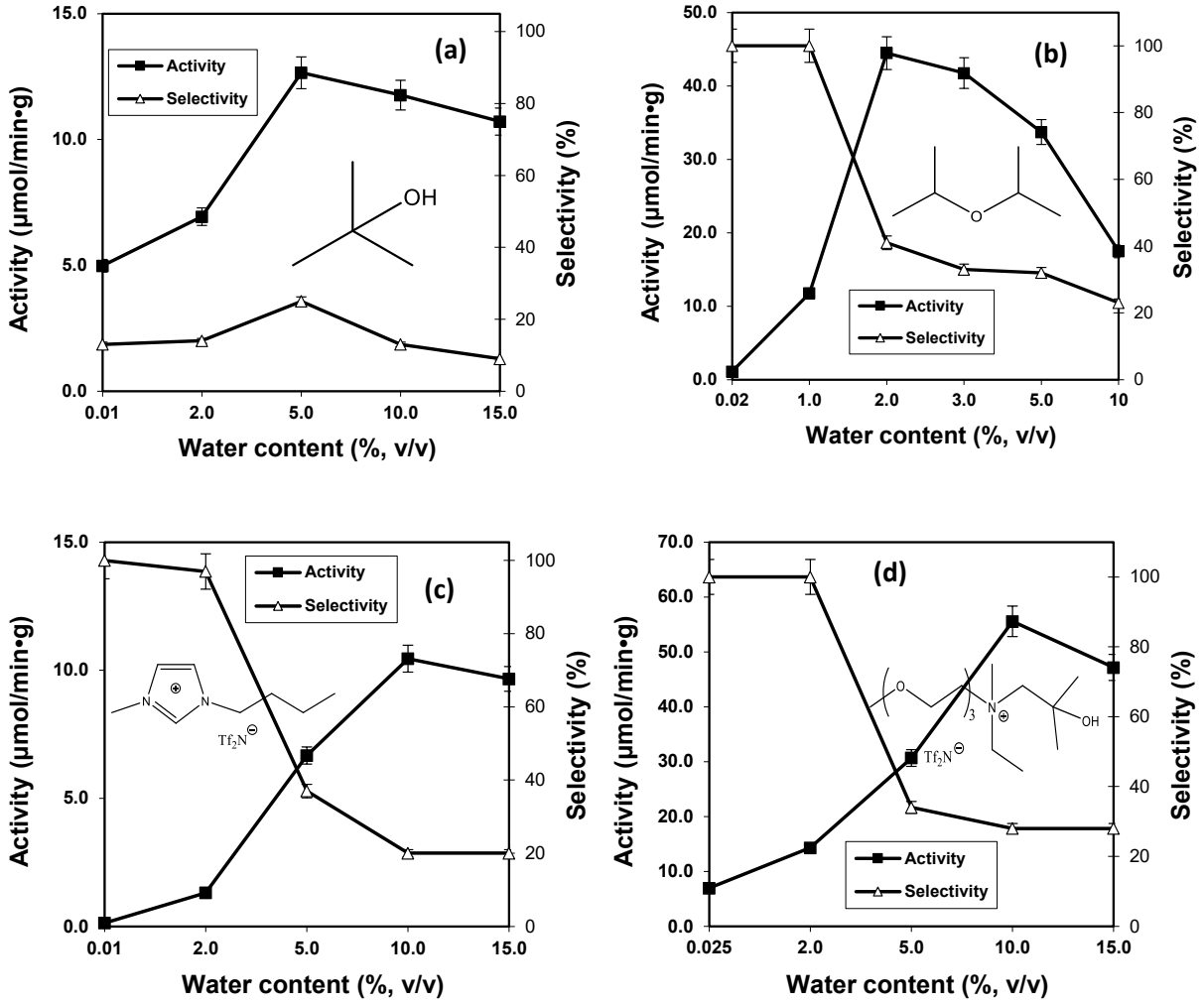


Figure 2 Effect of water content on subtilisin transesterification activity in different solvents: (a) *tert*-butanol, (b) diisopropyl ether, (c) [BMIM][Tf_2N] (**1**), and (d) IL **11**.

3.3. Effect of water on lipase and protease activities in water-mimicking ILs

A balanced water content is essential for achieving the optimum enzyme activity and selectivity during a transesterification reaction: too little water causes poor enzyme performance (i.e., low transesterification activity but high selectivity), and too much water speeds up the hydrolysis (*vs* synthesis) (i.e., low selectivity, and a competition of transesterification activity and hydrolysis activity). Lipase activities in Table 1 were obtained at a low-water content (~0.02 wt% water; this batch of Novozym 435 contains 1.06 wt% water based on our Karl-Fischer titration).

Furthermore, we systematically evaluated Novozym 435 activity in several top-performing solvents under different water contents (Figure 1 and Figure S17 in *Supplementary Information*). As illustrated in Figures 1(a) and 1(b), transesterification activity continued to decline with a higher water content (1.0–6.0 v%) in both *tert*-butanol and diisopropyl ether. Although the selectivity held high in diisopropyl ether, it gradually decreased with more water in *tert*-butanol. Non-functionalized [BMIM][Tf_2N] (**1**) showed the highest activity at 1 v% water while the selectivity consistently decreased with a higher water content (Figure 1(c)). However, our water-mimicking IL **11** (Figure 1(d)) allowed a linear increase of lipase activity with the water content (up to 2.0 v%) and a high selectivity at up to 4.0 v% water. Similar trends were seen for the other three "water-like" ILs **2**, **5** and **12** although the selectivities began to decrease after 2 v% water for **2** and **5** (Figure S17). The optimum lipase activities under different water contents are further compared in Table 2 for these selected solvents. Water-mimicking ILs **2**, **5**, **11** and **12** all enabled very high transesterification activities (11–12 μmol min^{-1} g^{-1} free CALB) under 2–3 v% water, which are up to 1.8-fold of that in *tert*-butanol, up to 1.6-fold of that in diisopropyl ether. A likely explanation for lipase activation in these dual-functionalized ILs is that the addition of a small amount of water creates a more "water-like" environment for the enzyme to increase the enzyme's flexibility and dynamics; since water molecules preferentially interact with *tert*-alcohol and glycol ether groups in ILs via hydrogen bonds, they are less likely to act as the substrate to cause ester hydrolysis. A further enzyme stabilization can be explained by the role glycol chain. It is reported that glycol chains in ether-functionalized ILs exhibit higher flexibility than rigid alkyl chains [61], and the grafting of glycol chain minimizes the intermolecular correlation (e.g., tail-tail segregation) and cation-anion specific interactions [62]. Another study [63] indicated that imidazolium and pyridinium cations interact with poly(ethylene glycol) 800 or 1000 through ion-dipole interaction

to form stable complexes; poly(ethylene glycol) molecules wrap around the cations. Therefore, water-mimicking cations could preferentially interact with the glycol chain, reducing the cation-protein interaction.

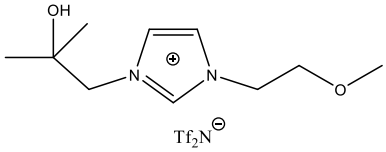
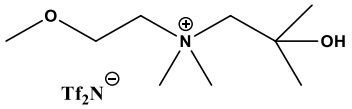
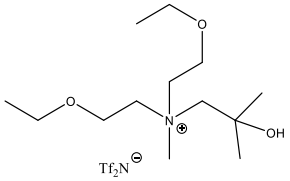
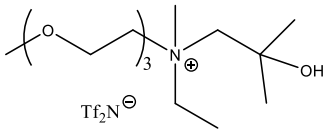
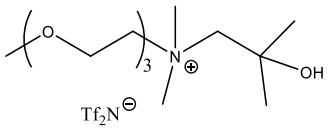
Furthermore, we extended these water-mimicking ionic solvents to examine another hydrolase known as subtilisin (a protease) using the transesterification of *N*-acetyl-L-phenylalanine ethyl ester with 1-propanol (see procedures in Section 4 of *Supplementary Information*). Figures 2 and S18 suggest that high protease activities and selectivities were not usually achieved under the same water content since the selectivity drastically decreased at a water content above 1–2 v%. Subtilisin showed the highest activity (12.65 μmol min^{-1} g^{-1} free protease) in *tert*-butanol with 5 v% water, but the selectivity was low across the entire water content range (Figure 2(a)). Diisopropyl ether with 2.0 v% water afforded a very high transesterification activity of 44.47 μmol min^{-1} g^{-1} free protease, but the selectivity was 41%. [BMIM][Tf_2N] (**1**) and water-mimicking ILs (**2**, **5**, **11**, and **12**) showed a similar pattern that highest activities appeared at 10.0 v% water, but the selectivities were below 30% (Figures 2 and S18). Table 2 compares the highest subtilisin activities and/or selectivities under different water contents for all solvents. The highest transesterification activity (55.55 was μmol min^{-1} g^{-1} free protease) was observed in IL **11** with 10.0 v% water (28% selectivity), which was 1.25-fold of the highest activity in diisopropyl ether (44.47 μmol min^{-1} g^{-1} free protease at 2 v% water). The highest subtilisin activity (14.28 μmol min^{-1} g^{-1} free protease) along with a high selectivity (100%) was also found in IL **11** with 2.0 v% water, which represents 1.2-fold of the activity (11.76 μmol min^{-1} g^{-1} free protease) with a high selectivity (100%) obtained in diisopropyl ether with 1.0 v% water. In summary, water-mimicking ILs could activate subtilisin by up to 1.2 fold while maintaining 100% selectivity when compared with diisopropyl ether.

3.4. Fluorescence emission spectra of enzymes in ILs

Fluorescence emission spectra provide valuable structural information of proteins in solvents. In aqueous buffer (pH 7.5, 20 mM), CALB and subtilisin exhibited emission maxima at 315 nm and 306 nm respectively (Figures S19(a) and S20(a) in *Supplementary Information*) due to tryptophan and tyrosine residues. In hydrophobic ILs, we dispersed 20 μL aqueous enzyme (20 mg/mL free CALB or subtilisin) in 1.0 mL ionic solvent. Because aqueous enzyme droplets were dispersed rather than homogeneously dissolved in ILs, fluorescence intensity may not match up that in aqueous buffer. Therefore, the shift in emission maximum could be more meaningful than the intensity. Generally, a red shift in emission maximum signifies the protein denaturation [64]. Table 3 derives emission maximum values for both CALB and subtilisin from fluorescence emission spectra in Figures S19 and S20. Both hydrolases displayed considerable red shifts (18–30 nm) of emission maximum in imidazolium-based IL **2** although this IL has been shown highly compatible with both enzymes. CALB in both ILs **8** and **11** exhibited emission maximum wavelengths (320 and 310 nm respectively), which resemble that in buffer (315 nm). Emission maxima of subtilisin in ILs **5** (310 nm) and **8** (317 nm) are also similar to that in buffer (306 nm), while the emission maximum in IL **11** suggests a red shift (341 nm). Fluorescence emission spectra in all ILs have much weaker intensity than in buffer (see Figures S19 and S20) due to: (a) dispersion rather than dissolution of enzymes in ILs, (b) the interference of ILs with the fluorescence signals. As a result, CALB in ILs **5** and **12**, and subtilisin in IL **12** were completed quenched to generate fluorescence spectra. It has been reported [65, 66] that imidazolium and ammonium ILs could significantly quench the fluorescence emission signals, not necessarily due to the complete denaturation of enzymes. Our fluorescence emission spectra indicate that both

hydrolases maintained characteristic emission maximum values in most water-mimicking ILs examined although the interference of ionic solvents on fluorescence signals is apparent.

Table 3 Fluorescence emission maximum of enzymes in different solvents

Solvent	Maximum wavelength (nm) of Free CALB	Maximum wavelength (nm) of Subtilisin
Phosphate buffer (pH 7.5, 20 mM)	315	306
2 	345	324
5 	___ [a]	310
8 	320	317
11 	310	341
12 	___ [a]	___ [a]

Note: [a] No fluorescence emission spectrum was detected.

4. Conclusions

Sixteen water-mimicking ILs were synthesized to carry glycol ether, *tert*-butanol, and/or trimethylsilyl groups. These functionalized ILs were designed to have low viscosities near 100 mPa s at 30 °C (as low as 70–80 mPa s) and high thermal stability (T_{dcp}) around 300 °C. These "water-like" ionic solvents are fully tailorable to become highly compatible with hydrolases. Especially in the presence of 2–3% water, several ILs (e.g. **2**, **5**, **11** and **12**) could activate CALB up to 1.8-fold of the activity in *tert*-butanol and up to 1.6-fold of the activity in diisopropyl ether.

Subtilisin could be activated by these water-mimicking solvents by up to 1.2-fold (with 100% selectivity) when compared with diisopropyl ether. Fluorescence emission spectra confirmed the characteristic emission maximum being retained in "water-like" ILs in most cases. Our approach has enabled a new direction for designing nonaqueous solvents that behave like water and are highly compatible with enzymes. Standard enzymatic transesterification reactions were used in the present study to evaluate the compatibility of functionalized ILs with hydrolases, but our undergoing project is to extend this biocatalysis process to asymmetric carbon–carbon bond formation reactions (such as Michael addition, Friedel-Crafts alkylation, and the aldol, Mannich, Morita–Baylis–Hillman, Henry, and Diels-Alder reactions), which will produce important chiral pharmaceutical ingredients [67-71].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at xxxxxx.

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