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Chemoselective Nitration of Phenols with *tert*-Butyl Nitrite in Solution and on Solid Support

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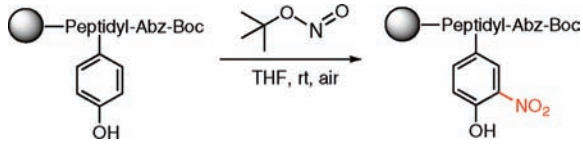
ABSTRACT



***tert*-Butyl nitrite was identified as a safe and chemoselective nitrating agent that provides preferentially mononitro derivatives of phenolic substrates in the presence of potentially competitive functional groups. On the basis of our control experiments, we propose that the reaction proceeds through the formation of *O*-nitrosyl intermediates prior to *C*-nitration via homolysis and oxidation. The reported nitration method is compatible with tyrosine-containing peptides on solid support in the synthesis of fluorogenic substrates for characterization of proteases.**

The technique of fluorescence resonance energy transfer (FRET) is currently a common method for monitoring structural, functional, or aggregation changes in evaluated biomolecules.[1] Real-time monitoring of hydrolase activity is a particularly useful application of FRET because the substrate can be chemically modified at sites remote to the scissile bond, minimizing probe interferences with substrate recognition. The 2-aminobenzoic acid (2-Abz) fluorophore and 3-nitrotyrosine quencher pair has been utilized extensively in determining proteolytic activities of endoproteases.[2] As amino acids, these agents represent a convenient fluorophore−quencher combination that can be readily incorporated into a conventional solid-phase peptide synthesis protocol. As a part of a program in evolution of site-specific proteases as therapeutic agents, we are interested in developing a flexible and practical platform for kinetic and thermodynamic characterization of evolved enzymes. Thus, we envisioned a unified synthetic strategy for accessing both internally quenched substrates, useful for real-time kinetic analysis of active enzymes, as well as unquenched variants, suitable for fluorescence quenching and anisotropy studies with inactive mutants. We were very keen, therefore, to identify a reagent that could perform a nitration reaction on solid-phase-immobilized peptides containing tyrosines with high efficiency and selectivity. Unfortunately, the existing nitrating agents, such as mixed acids, superacids, and metal nitrates, are not suitable for polymer-supported substrates because of their polymer-reactive or heterogeneous nature.[3,4] In addition, many of the existing reagents can lead to overnitration of phenols[4a,g,h] and other unwanted reactions with a variety of functional groups found in peptides.[5] Thus, development of a methodology enabling chemoselective nitration of polymer-immobilized tyrosine would be valued.

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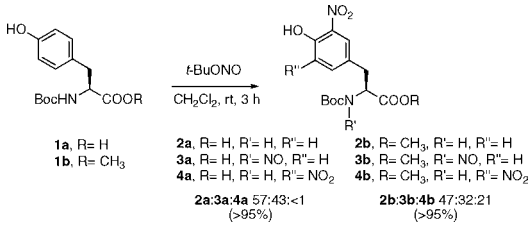
We disclose in this paper a novel nitration procedure using *tert*-butyl nitrite, a reagent that displays exquisite chemoselectivity for phenols, yields only *tert*-butyl alcohol as a byproduct, and is relatively safe, volatile, and soluble in a variety of organic solvents.

In our attempt to perform diazotization of tyrosine with *t*-BuONO,[6] we observed the formation of unexpected yellow products. To explore this finding further, we subjected Boc-Tyr-OH (**1a**) to the nitrite treatment in dichloromethane at room temperature (Scheme 1). After 3 h of agitation, we

Scheme 1. Nitration of Tyrosine Derivatives with *t*-BuONO



detected a complete consumption of **1a** and quantitative (>95%) formation of Boc-Tyr(3-NO_2)-OH (**2a**) and corresponding *N*-nitroso derivative **3a** (**2a**:**3a** 57:43). While there have been reports of *C*-nitro products emerging from the exposure of electron-rich aromatic compounds to inorganic[7] or organic[8] nitrites, no mechanistic or scope studies have been carried out. In ^{1}H NMR spectrum of the crude reaction mixure we also identified a trace ammount of bisnitrated product **4a** (<1%), and we confirmed that both **3a** and **4a** can arise from **2a** by re-exposing the latter to a larger excess of *t*-BuONO. The presence of a nitro rather than a nitrosyl group in **2a** was corraborated by mass spectrometry, ^{1}H NMR,[9] and UV−vis spectroscopy ($\lambda_{max} \approx 430$ nm). Although *N*-nitrosyl compound **3a** rapidly decomposed upon isolation to form **4a** along with several other compounds, we confirmed its structure by comparison of ^{1}H NMR resonances with those of the more stable ester variant **2b**, prepared from Boc-Tyr-OMe (**1b**) along with **3b** and **4b** (**2b**:**3b**:**4b** 47:32:21, >95%) under identical conditions (Scheme 1). On the basis of these results, we speculated that a careful optimization of reaction conditions could provide a selective mononitration procedure.

To identify a more practical nitration procedure, we proceeded to screen a variety of solvents (Table 1). While

Table 1. Optimization Studies of the Reaction between **1a** and *t*-BuONO[a]

			yield[b] (%)		
entry	solvent	time (h)	**2a**	**3a**	**4a**
1	MeOH	16	no reaction		
2	$CHCl_3$	3	>95	nd	nd
3	$CHCl_3$	16	19	50	31
4	DMSO	3	43	nd	nd
5	DMSO	16	>95	nd	nd
6	DMF	3	>95	nd	nd
7	DMF	16	58	16	25
8	acetonitrile	1.5	66	23	10
9	acetonitrile	16	37	37	24
10	diethyl ether	16	68	nd	nd
11[c]	diethyl ether	3	95	nd	nd
12	THF	1	93	nd	nd
13	THF	3	>95	nd	nd
14	THF	6	95	nd	4
15	THF	16	85	nd	14

[a] Unless specified otherwise, all reactions were carried out at room temperature in 0.2 M solutions of **1a** in THF with 3 equiv of *t*-BuONO. [b] All yields were measured by ^{1}H NMR. [c] Under reflux conditions, nd = not detected.

protic solvents appeared to prevent nitration altogether (entry 1), use of chloroform noticeably improved the chemoselectivity of this reaction (entry 2). Upon prolonged exposure, however, undesired byproducts **3a** and **4a** became dominant (entry 3), limiting the utility of this solvent. In DMSO, the reaction was noticeably slower than in the halogenated solvents but provided exclusively the desired product **2a** (entries 4 and 5). The reaction proceeded in DMF at a similar rate as in chloroform (entry 6) but displayed higher chemoselectivity for mononitration, since byproducts **3a** and **4a** emerged only upon extended incubation with *t*-BuONO (entry 7). The use of acetonitrile, on the other hand, severely compromised chemoselectivity of the reaction with byproducts emerging early in the reaction course (entry 8) and accumulating significantly after 16 h (entry 9). While the use of diethyl ether led to a relatively slow nitration progress (entry 10), a temperature increase led to nearly quantitative conversion to **2a** in 3 h (entry 11). Finally, by employing THF we obtained a nitration procedure that is both highly selective and reasonably fast. Within 1 h, reaction conversion reached 93% (entry 12) and became nearly quantitative after 3 h (entry 13). While overnitration product **4a** started to form after a 6-h incubation (entry 14), no detectable *N*-nitrosylated product emerged even after 16 h (entry 15). These results demonstrate that the rates of neither the desired *C*-nitration nor the undesired *N*-nitrosylation and bisnitration reactions appear to correlate with solvent polarities. Overnitration has occurred in both polar and nonpolar solvents, albeit at significantly distinct rates: faster in dichloromethane ($\varepsilon = 8.9$[10]) and acetonitrile ($\varepsilon = 36.6$), but slower in DMF ($\varepsilon = 38.3$)

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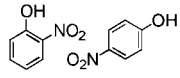
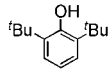
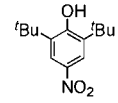
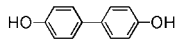
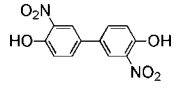
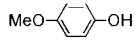
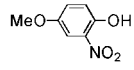
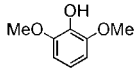
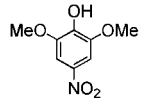
and THF (ε = 7.5). Widely varying ratios of the byproducts (cf. entries 3, 7, 8, and 15) suggest distinct mechanistic pathways responsible for their formation. The preference for *N*-nitrosylation in acetonitrile could be rationalized by the formation of a solvent−nitrosonium adduct,[11] capable of the subsequent *N*-nitrosylation of the carbamate. No byproducts were observed in the aprotic solvents at the extremes of polarity: DMSO (ε = 47.2) and diethyl ether (ε = 4.3). Unlike these solvents, however, THF maintained a robust nitration rate, providing a procedure that was fast, selective, and more suitable for solid-phase synthesis, considering favorable swelling properties of cross-linked polystyrene resins in THF.[12]

To establish the scope of this reaction, we exposed a series of phenols to *t*-BuONO in THF. We found that this procedure was effective at converting electron-rich compounds in a variety of steric contexts into *C*-nitro derivatives (Table 2).

Table 2. Nitration on Phenols with *t*-BuONO[a]

Aromatic alcohol —*t*-BuONO, THF→ Product

entry	compounds	time	product	Yield[b] (%)
1		2 h		45, 37[c]
2		12 h		72
3[d]		16 h		82
4		15 min		78
5		10 min		85

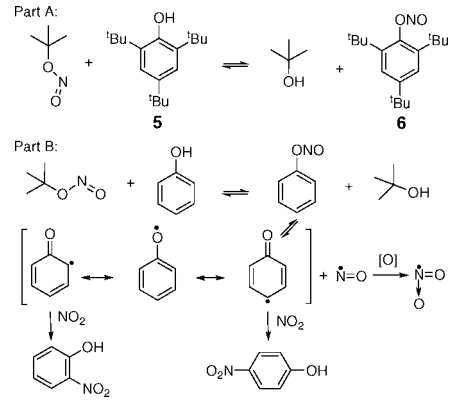
[a] Unless specified otherwise, all reactions were carried out at room temperature using 3 molar equiv of *t*-BuONO and 5 mL of solvent per mmol of phenol. [b] Isolated yields, unless specified otherwise. [c] ^{1}H NMR yields (18% starting material recovered). [d] 6 equiv of reagent.

When both *ortho* and *para* positions are available, a mixture of mononitrated regioisomers was obtained (entry 1). The resistance to overnitration in THF was also confirmed by the lack of reactivity of both *o*- and *p*-nitrophenols toward *t*-BuONO (1.5 equiv, 2 h). As expected, *ortho*- and *para*-substituted phenols (entries 2 and 3) provided exclusively the corresponding *para*- and *ortho*-nitro derivatives, respectively. Notably, single regiochemical outcomes with 4-methoxyphenol and 2,6-dimethoxyphenol as substrates (entries 4 and 5) highlighted a dominant role of hydroxyl groups over alkoxy substituents in directing the reaction regiochemistry.

Next, we determined the extent of chemoselectivity displayed by *t*-BuONO with respect to functional groups present in peptides. Gratifyingly, aromatic ethers (anisole, Ac-Tyr(Bn)-OMe) and *N*-formylindole (Ac-Trp(CHO)-OMe) were unreactive toward the organic nitrite (3 equiv of *t*-BuONO, THF, 25 °C, 24 h) confirming the strict requirement for an aromatic hydroxyl group in this reaction. In addition, no nitrosylation of primary or secondary amides was detected upon prolonged incubation (>6 h) of Ac-Ala-OMe and Ac-Gln-OMe. Of all functionalities tested, only the sulfide-containing compounds Boc-Cys(Tr)-OH and Boc-Met-OH yielded complex mixtures, as seen in earlier diazotization attempts of related compounds.[13]

To unravel the role that the phenolic hydroxyl group plays in this nitration, we subjected 2,4,6-tri-*tert*-butylphenol **5** to a large excess of *t*-BuONO (Scheme 2, part A). Within 10 min, we observed complete consumption of **5** and concomitant formation of a new compound that we have assigned as *O*-nitroso derivative **6** on the basis of ^{1}H NMR and FTIR data. Furthermore, in *t*-BuOH as a solvent, **6** reverted to **5** with concomintant formation of *t*-BuONO, confirming both the reversibility of the *O*-nitrosylation process and the chemical nature of **6** as aromatic nitrite. Upon storing at room temperature in $CDCl_3$, **6** slowly transformed into a complex mixture of *C*-nitro derivatives[14] through apparent intermediacy of a phenoxyl radical of **5**, detected by its characteristic blue color.[15] The isolation of the *O*-nitroso species, the lack of reactivity in protic solvents, and the reaction kinetics—first order with respect to both phenol and *t*-BuONO (see the Supporting Information)—allowed us to propose that the reaction is initiated by a rate-limiting nitrosyl exchange between the alkyl nitrite and aromatic hydroxyl groups

Scheme 2. Mechanistic Analysis and Proposed Pathway for the Nitration Reaction with *t*-BuONO



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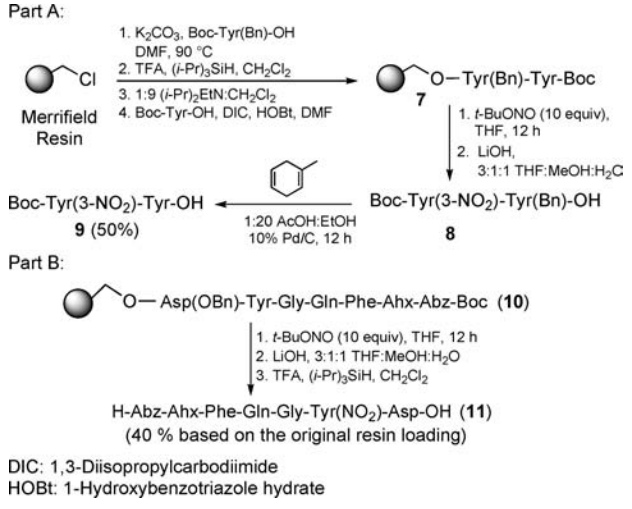
(Scheme 2, part B), as shown previously for aliphatic alcohols.[16] The aromatic *O*-nitroso derivatives undergo subsequent thermal homolysis to furnish resonance-stabilized phenoxyl radicals and nitric oxide.[17] Our attempts to detect any *C*-nitroso intermediates failed, and we, therefore, propose that the final steps of this reaction involve rapid oxidation of nitric oxide with molecular oxygen,[18] prior to coupling of the resultant nitrogen dioxide with phenoxyl radicals.[19] A crossover experiment with **6** as a nitrating reagent furnished the mononitro product (4-methoxy-2-nitrophenol) with 4-methoxyphenol as a substrate (see the Supporting Information), confirming the intermediacy of aromatic nitrites in this process. Use of anisole in this experiment, however, left the aromatic ether unmodified, establishing that the formation of phenoxyl radicals is the prerequisite for the observed *C*-nitration. Participation of phenoxyl radicals has been postulated for nitration reactions mediated by tetranitromethane[5a] and peroxynitrite[20] in polar protic media, on the basis of isolation of biaryl ethers and biphenols as dominant byproducts. We do not observe these products, presumably due to the improved solvation of phenoxyl radicals in the aprotic solvents, making the bimolecular cross-coupling less likely.

Having developed an efficient nitration procedure for solution-phase transformations, we sought to implement this methodology on polymer-supported phenolic substrates. We generated test dipeptide **7**, containing both protected and unprotected tyrosine residues on Merrifield resin (Scheme 3, part A) to investigate the ability of the reagent to target selectively the phenol functional group in the presence of carbamate, secondary amide, and aromatic ether functionalities. The sole product isolated after the treatment of **7** with *t*-BuONO in THF and subsequent hydrolysis was Tyr(3-NO_2)-containing derivative **8**. Finally, we accomplished efficient debenzylation of **8** in the presence of a nitro group under transfer hydrogenation conditions to provide Boc-Tyr(3-NO_2)-Tyr-OH (**9**).

Scheme 3. On-Bead Nitration and Synthesis of a Fluorogenic Peptide



Our interest in tobacco etch virus protease (TEV-Pr) as a scaffold for the evolution of enzymes with novel properties prompted us to explore synthetic approches toward fluorogenic substrates of TEV-Pr. In a search for the shortest TEV-Pr substrate, a tripeptide truncate (FQG) of the optimal linear epitope ENLYFQG,[21] flanked by the *C*-terminal Asp-Tyr dipeptide and *N*-terminal 6-aminohexanoic acid spacer-fluorophore (Ahx-Abz) unit (**10**), was produced as a test compound for the solid-phase nitration procedure (Scheme 3, part B). Reaction of **10** with *t*-BuONO, followed by hydrolysis, yielded a selectively mononitrated peptide furnished with a 3-nitrotyrosine chromophore in >90% purity, as detected by reversed-phase HPLC, spectrophotometric analysis, and mass spectrometry (see the Supporting Information). While the truncated substrate was not recognized by TEV-Pr, this stringently quenched peptide ($F_0/F_\infty \approx 0.9\%$) was processed effectively by chymotrypsin, as detected in real time through the release of a fluorescent anthranilamide-containing fragment (see the Supporting Information).

In conclusion, we have presented here a novel procedure for selective and efficient nitration of phenols in aprotic media, which is compatible with polymer-supported substrates. We expect that the potential of this methodology to provide access to both internally quenched and unquenched peptides will greatly facilitate characterization of novel proteolytic activities. Finally, the facile and selective introduction of a nitro group described herein may also initiate other synthetic opportunities, such as late-stage chromophore installation and formation of photolabile groups.

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Supporting Information Available: Experimental procedures and compound characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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