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Practical and Selective Syntheses of *S*-Acyl and *N*-Acyl Glutathiones with *N*-Acyl Imidazoles in Water

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A selective, mild, convenient, and green protocol for the preparation of *S*-acyl and *N*-acyl glutathiones is described involving the chemical modification of glutathione (GSH) with *N*-acyl imidazoles at room temperature in water. The syntheses of *S*-acyl glutathiones were achieved in very high yields using 1 equiv. of an *N*-acyl imidazole in water at room temperature, without the need of a base. Double acylation of GSH with various *N*-acyl imidazoles in weakly basic aqueous media in the presence of *N*-hydroxysuccinimide (HOSu) as the activating reagent followed by selective deprotection of the *S*-acyl group with aqueous ammonia at room temperature gave high yields of *N*-acyl glutathiones. Moreover, the reaction could accommodate a diverse range of carboxylic acids such as (hetero)benzoic acids, phenylacetic acids, aliphatic acids from short-chain fatty acids (including acetic acid), long-chain polyunsaturated fatty acids, secondary or tertiary amino acids, and carboxylic acids containing clickable functional groups, fluorescent probes, or drugs.

Introduction

The efficient and selective synthesis of novel *S*-acyl and *N*-acyl glutathione (GSH) derivatives has captivated rising interest due to their potential therapeutic effects/properties in treating Alzheimer's disease, Parkinson's disease, liver disease, viral diseases, cancer, cardiac diseases, and diabetes.[1] In addition, they are promising agents for GSH restoration, not only because they are more stable in plasma and cell-penetrating than GSH, but they are also easily converted into GSH (and the corresponding carboxylic acid) by intracellular hydrolases.[2] Indeed, naturally occurring GSH (γ-L-glutamyl-L-cysteinyl-glycine), the main redox regulator in cells, cannot easily cross the phospholipid bilayer due to its unique structure (GSH exists as a zwitterion in aqueous solution since the glutamic acid and the cysteine residues are linked from the γ-carboxyl group of glutamic acid) thus restricting its use as a therapeutic agent.[3] Permeability is essential to cells' ability to obtain nutrients, maintain and restore a stable interior environment and redox homeostasis. Acylated analogs are privileged scaffolds in medicinal chemistry that are used to improve physicochemical/pharmacokinetic properties. Acylating agents provide a convenient method for installing, among others, linkers that enable bioconjugation or fluorescence.

The chemoselective acylation of the highly reactive (nucleophilic) thiol moiety of the cysteine residue of GSH has been widely reported,[4] utilizing (a) acid anhydride in the presence of triethylamine in organic solvents,[4a] (b) acyl chlorides in trifluoroacetic acid,[4b] and, more recently, (c) preformed *N*-acyl benzotriazoles, synthesized from carboxylic acids using the thionyl chloride procedure, in the presence of potassium bicarbonate (1 equiv) in aqueous methanol.[4c] Conversely, reported methods to synthesize derivatives of *N*-acyl glutathiones remain underdeveloped and require smart manipulation of protecting group chemistry due to the greater reactivity of thiols over amines under standard reaction conditions.[5] That said, the following methods are worth mentioning: (a) *N*-acylation of glutathione disulfide (GSSG) with an activated ester followed by S–S reduction;[5a] (b) use of a trityl group to protect the thiol group of GSH, then *N*-acylation followed by trityl group deprotection;[5b] (c) use of 4-nitrobenzoyl to protect the thiol group of GSH, then *N*-acylation with 1-acyl-1*H*-benzotriazole followed by deprotection using pyrrolidine in dry tetrahydrofuran (THF)-methanol for 4 h;[4c] and (d) a two-step synthesis of *N*-butanoyl-GSH using butyric anhydride, followed by selective thioester methanolysis of the *S,N*-dibutanoylated product.[5c] However, these methods have strong limitations for large-scale applications because the reagents are highly corrosive and toxic, environmentally damaging solvents are necessary, and difficulties in the purification of the final *S*- and *N*-acyl glutathiones are usually encountered.

N-Acyl imidazoles are easily accessible, highly water soluble, activated carboxylic acid derivatives that have a relatively long half-life (cf. acyl chlorides, which were widely used in previous studies). These reagents can be prepared from reactions between abundant carboxylic acids and *N,N'*-carbonyldiimidazole (CDI).[6] CDI is not only an inexpensive feedstock chemical, but it is synthetically easy to work with, generates readily removable byproducts, does not require an additional base, and is commonly employed in industrial settings.[7] Since no

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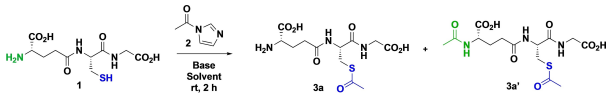
dehydrating agent is needed in this process and the byproducts are CO_2 and water-soluble imidazole, *N*-acyl imidazoles can be isolated through a simple extractive workup without chromatographic purification or directly used for the amide and ester bond formation under mild conditions, when prepared with a water-miscible solvent. In addition, thanks to their chemical selectivity and water stability, *N*-acyl imidazole reagents can be uniquely employed in chemical biology research, including chemical modification of proteins/peptides.[8] To the best of our knowledge, the use of CDI in the formation of *N*-acyl glutathiones has not been reported yet.

In this context, we decided to study *N*-acyl imidazoles as a nonacidic acylating agent for both the selective preparation of *S*-acyl and *N*-acyl glutathiones in aqueous media. In this work, we report the development of this methodology and its successful application to a wide range of acyl imidazoles, from short-chain fatty acids (including acetic acid) to long-chain polyunsaturated fatty acids, aromatic acids, and acids containing a handle for bioconjugation or fluorescence.

Results and Discussion

Because of the high solubility of GSH and *N*-acyl imidazole in water, we decided to initially treat GSH with readily available *N*-acetyl imidazole to selectively synthesize the industrially important bioactive compound *S*-acetyl-L-glutathione[9] in weakly basic aqueous solution at room temperature. Multiple experiments were performed to screen for an effective base and the proper molar equivalents; the results are presented in Table 1.

Table 1. Optimization of the reaction conditions for the preparation of *S*-acetyl-L-glutathione (**3a**).[a]



Entry	Base	Equiv of base	Solvent (0.25 M)	Ratio[b] of **1**:**3a**:**3a'**
1	DABCO	1.2	1:3 CH_3CN/H_2O	22:56:22
2	DMAP	1.2	1:3 CH_3CN/H_2O	7:77:16
3	TEA	1.2	1:3 CH_3CN/H_2O	5:68:27
4	DBU	1.2	1:3 CH_3CN/H_2O	3:94:3
5	Py*HCl	1.2	1:3 CH_3CN/H_2O	0:96:4
7	Na_2CO_3	2.5	1:3 CH_3CN/H_2O	0:75:25
8	$KHCO_3$	1	1:3 CH_3CN/H_2O	30:50:20
9	/	/	1:3 CH_3CN/H_2O	26:64:10
10	/	/	H_2O	80:20:0
11	/	/	1:4 THF/H_2O	0:100 (98)[c]:0

[a] Unless otherwise noted, all reactions were carried out with GSH (**1**, 76.7 mg, 0.25 mmol, 1 equiv) and *N*-acetyl imidazole (**2**, 132.1 mg, 0.3 mmol,1.2 equiv). [b] Ratio determined by peak areas in LC/UV/MS. [c] Isolated yield after treatment of the reaction with $KHSO_4$ (3 N) and precipitation with acetone as the cosolvent.

Using 1:3 acetonitrile/water as the solvent, all basic conditions tested afforded a mixture of mono- and double-acylated products **3a** and **3a'**, respectively, with moderate-to-good conversion but without selectivity control (Table 1, entries 1–8). Eventually, without the addition of a base and switching the reaction media from acetonitrile/water to THF/water, we were able to achieve full conversion together with complete control on the selectivity toward the formation of the mono-acylated product **3a** (Table 1, entry 11). Therefore, treatment of GSH (**1**) with *N*-acetyl imidazole (**2**, 1 equiv) at 25 °C in 1:3 THF/water gave exclusively *S*-acyl GSH (**3a**) in 98% yield in only 45 min.

Although several stability studies of various *N*-acyl imidazoles in acidic and basic solutions have already been published, the conditions employed were generally not those most suitable for the synthesis of acid- and base-sensitive compounds.[10] Indeed, in line with the results shown in Table 1, *N*-acetyl imidazole (**2**) is probably not very stable in basic aqueous environments, since its hydrolysis can be catalyzed by the anionic imidazole released (azolide) in the reaction and the mechanism involves a proton abstraction from a water molecule (i.e., the imidazole nucleofuge also serves as a base). In addition, in the absence of an external base, the acyl imidazole can first be protonated by the acidic thiol group to generate an ion pair, which has enhanced reactivity due to the proximity effect of the subsequent attack of the thiolate on the carbonyl, expelling imidazole through a formal nucleophilic acyl substitution mechanism.[11] Based on the GSH pK_a values (8.7, 9.2, 2.1, 3.5) and isoelectric point (5.9), the two carboxylates are deprotonated at the working pH (moderately acidic) and do not interact with the electrophile, avoiding, inter alia, the formation of unproductive mixed anhydrides. *N*-Acyl imidazoles are generally considered to be mild acylating agents, but their reactivity, like that of other acylating reagents, clearly depends on steric and electronic factors. Gratifyingly, many *N*-acyl imidazoles were found to be useful acylating agents in this protocol (Table 2). For example, different aliphatic acids could be employed in this reaction, allowing the preparation of thioesters **3b**–**h** with different alkyl chains in good-to-high yields.

In particular, other than using short-chain acids (**3b**, **3c**, and **3e**), this *S*-acylation of GSH with an *N*-acyl imidazole in water can also be applied to more complex acids such as 10-azidodecanoic acid (compound **3d**), allowing the introduction of a terminal azide, which is useful for biorthogonal reactions such as copper-catalyzed alkyne-azide cycloaddition, without further processing. A series of sterically demanding saturated and unsaturated fatty acids (stearic, oleic, and α-linolenic acid) was successfully employed, achieving compounds **3f**–**h** in good-to-excellent yields. Furthermore, various aromatic acids, bearing *para*-, *meta*-, or *ortho*-substituted electron-donating or electron-withdrawing groups, were demonstrated to be suitable electrophiles for this reaction, providing thioesters **3i**–**o** in good yields, regardless of the nature of the substituents. Also, furan-3-carboxylic acid, bearing a heterocyclic furan moiety, was determined to be a suitable coupling partner, as indicated by the synthesis of compound **3p**. To our delight, this method-

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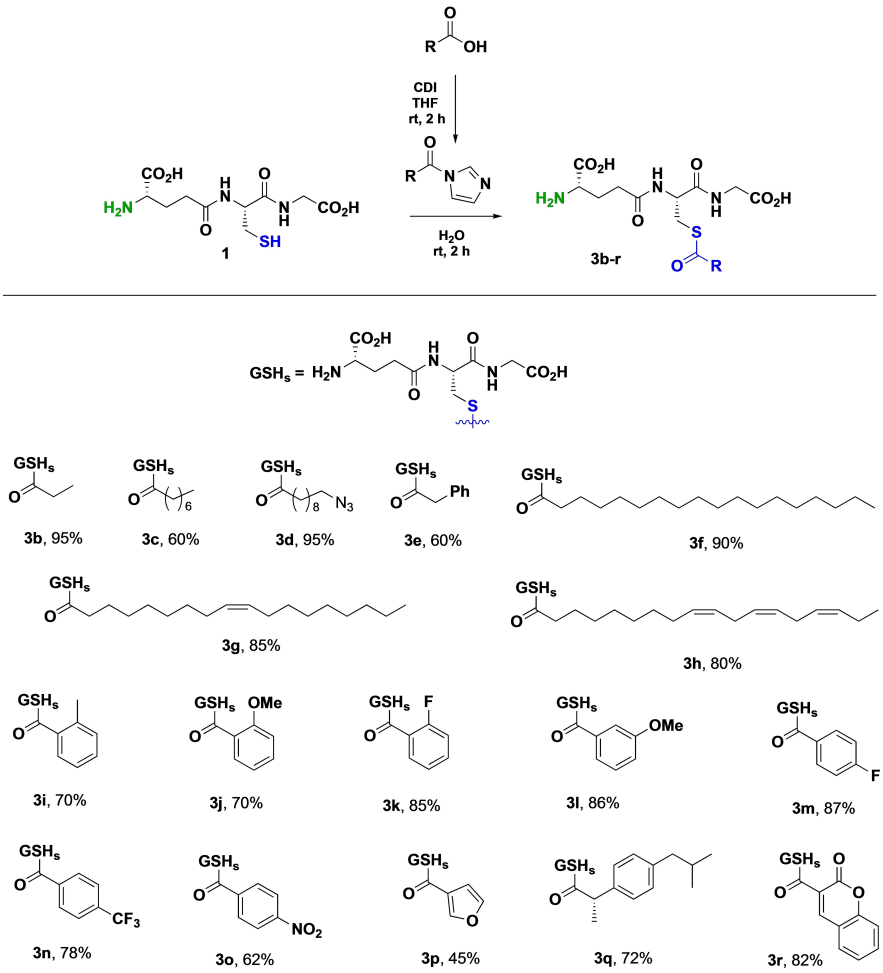
Table 2. Substrate scope investigation of the *S*-acylation of GSH with *N*-acyl imidazole in water.[a]



[a] Unless otherwise noted, all reactions were carried out with GSH (**1**, 307 mg, 1 mmol, 1 equiv) and an *N*-acyl imidazole (1.2 equiv). Isolated yields are reported.

ology could be extended to access a drug-GSH conjugate scaffold (ibuprofen-GSH derivative **3q**) using (*R*)-2-(4-isobutylphenyl)propanoic acid. Interestingly, and potentially very useful for the determination of GSH in biological fluids, the synthesis of a derivative with a fluorescent label was carried out. Coumarin-labeled **3r** was efficiently prepared from GSH with *N*-coumarin-3-carbonyl imidazole using our developed method.

The success of this chemoselective thioacylation over amidation can be explained by considering that the amine still forms a zwitterion with the adjacent carboxylic acid and is therefore not available for acylation, despite Yoshida *et al.* reporting that the byproduct imidazole (released during the reaction) can be employed to aid amide bond formation in the native chemical ligation reaction with the amino acid thioester.[12] Knowing that the thiol group is rather more nucleophilic than the amino group and no intra- or intermolecular S-to-N acyl transfer occurs even under basic pH conditions (competition hydrolysis of the thioester), we sought to synthesize the corresponding *S*-,*N*- doubly acylated GSH and to minimize mild thioester hydrolysis in order to selectively achieve *N*-acyl GSH derivatives. To do so, the contemporary deprotonation of the terminal zwitterionic amino group (to obtain the free nucleophilic amine) and the activation of the *N*-carbonyl imidazole derivatives (to avoid slow hydrolysis under the necessary basic conditions) would be needed to efficiently acylate the inherently least nucleophilic terminal amino group of GSH in water. It has been shown that carbonyl imidazole species can be engaged by Lewis base additives such as DMAP, DBU, and 1-hydroxybenzotriazole (HOBt), as an amidation step promoter/catalyst, through the nucleophilic displacement of imidazole.[13] These reports inspired us to consider that the imidazole anion generated by putative nucleophilic attack by DBU, or some other nucleophilic species, on the carbonyl imidazole might provide a path toward our aforementioned goal of double acylation. Our studies commenced with the attempted double acylation of GSH with commercially available *N*-benzoyl imidazole (Table 3). We observed intramolecular conversion of GSH to pyroglutamate (5-oxo-proline, also known as pidolic acid) during its derivatization with DBU, DMAP, or

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other bases, which were consequently discarded as additives (Table 3, entries 1–3). Conversely, higher performance was obtained with HOBt, and a higher selectivity on the double-acylated product **6** was obtained in the presence of sodium bicarbonate as a weak water-soluble base (Table 3, entries 4–6). HOBt provided consistent rate enhancements but presented some drawbacks, including a high cost per mole, highly energetic decomposition, potential for explosion, and transportation restrictions.[14]

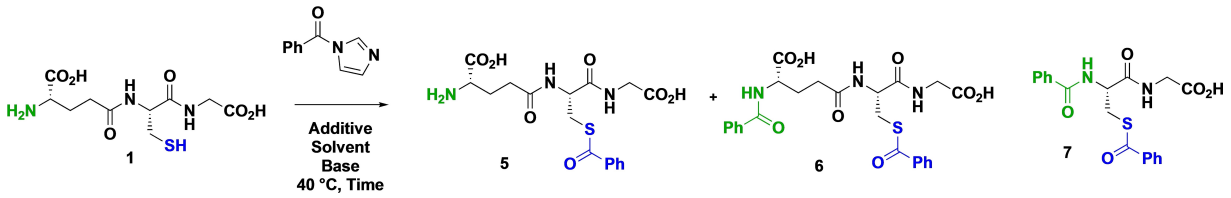
More recently, other catalysts with improved safety profiles have been introduced, including 2-hydroxy-5-nitropyridine,[15a] pyridinium hydrochloride,[15b] and hydroxylamine derivatives such as *N*-hydroxysuccinimide (HOSu).[15c] Unlike HOBt, these catalysts offer the advantages of low cost, ease of handling, and chemical stability. Among them (Table 3, entries 7–11), we were delighted to find that a stoichiometric amount (i.e., 2.5 equiv) of HOSu increased the rate of amide coupling, allowing full conversion to the double-acylated product **6** (Table 3, entry 10). It is likely that HOSu can protonate the acyl imidazole, enhancing its electrophilicity and promoting amide bond formation. In addition, the formation of a highly reactive OSu ester is not excluded. A further advantage of HOSu compared to HOBt is that HOSu can be easily removed during workup as it is water soluble even at acidic pH, in which the double-acylated product **6** precipitates. With the optimized conditions in hand, the selective synthesis of the desired *N*-acylated GSH derivatives occurred by directly subjecting the diacetylated intermediates, not isolated, to mild thioester ammonolysis using a 30% ammonia solution,[16] as reported in Table 4.

In particular, we explored the scope of this mild *N*-acylation reaction of GSH by using a variety of functionalized acyl imidazoles as the acylation reagent. Different substituted aromatic rings were well tolerated, regardless of the electronic character or position (*ortho*-, *meta*-, or *para*−) of the substituent, affording amides **8a**–**h** in good yields of up to 80%. Various aliphatic acids could also be employed in this reaction, allowing the preparation of amides **8i**–**n** with different alkyl chains, including the bulky *t*-Bu group, in yields ranging from 60% (**8i** and **8k**) to 90% (**8l**). Remarkably, using this protocol, we were also able to synthesize the tetrapeptide **8n** using *N*-protected L-proline as the precursor of the acylating agent in modest yield.

Conclusions

In conclusion, we have developed facile and efficient methods for selective syntheses of both *S*-acyl glutathiones and *N*-acyl glutathiones in good-to-high yields under mild reaction conditions from readily available acyl imidazoles using water as the solvent. These acylation reactions, which represent a better complement to existing technologies, are highly practical as they (i) only involve readily available, inexpensive, and relatively safe reagents, (ii) utilize water as the solvent, (iii) can be performed on a multigram scale, and (iv) can be used on carboxylic acids directly by *in-situ* formation of the acyl imidazole electrophile. Moreover, a wide tolerance of aromatic and aliphatic acids, bearing different substituents or alkyl chains, has been demonstrated. Furthermore, this approach is compatible with a wide range of functionalized carboxylic acids, such as benzoic acids, phenylacetic acids, and aliphatic acids. Thus, we believe that this mild and practical method will find

Table 3. Optimization of the reaction conditions for the double benzoylation of L-glutathione (**6**).[a]



Entry	Additive	Equiv of additive	Base	Equiv of base	Solvent (0.1 M)	Time (h)	Ratio[b] of **1:5:6:7**
1	/	/	$KHCO_3$	2.1	4:1 H_2O/THF	24	0:95:5:0
2	DBU	0.5	$KHCO_3$	2.1	4:1 H_2O/THF	6	0:2:3:95
3	DMAP	0.5	$KHCO_3$	2.1	4:1 H_2O/THF	6	0:25:25:50
4	HOBt	1.1	$KHCO_3$	2.1	4:1 H_2O/THF	6	10:55:35:0
5	HOBt	3	$KHCO_3$	2.1	4:1 H_2O/THF	6	0:10:70:20
6	HOBt	2.1	$KHCO_3$	2.1	4:1 H_2O/THF	2	0:3:97:0
7	Py*HCl	1.1	$KHCO_3$	2.1	4:1 H_2O/THF	6	20:40:40:0
8	2-PAM[c]	2.1	$KHCO_3$	2.1	4:1 H_2O/THF	6	10:85:5:0
9	HOSu	2.1	$KHCO_3$	2.1	4:1 H_2O/THF	2	0:4:96:0
10	HOSu	2.5	$KHCO_3$	2.5	4:1 H_2O/THF	2	0:0:100:0
11	HOSu	1.1	$KHCO_3$	2.5	4:1 H_2O/THF	16	10:30:40:20

[a] Unless otherwise noted, all reactions were carried out with GSH (**1**, 76.7 mg, 0.25 mmol, 1 equiv) and *N*-benzoyl imidazole (**4**, 430.4 mg, 2.5 mmol, 2.5 equiv). [b] Ratio determined by peak areas in LC/UV/MS. [c] 2-pyridine aldoxime methyl chloride.

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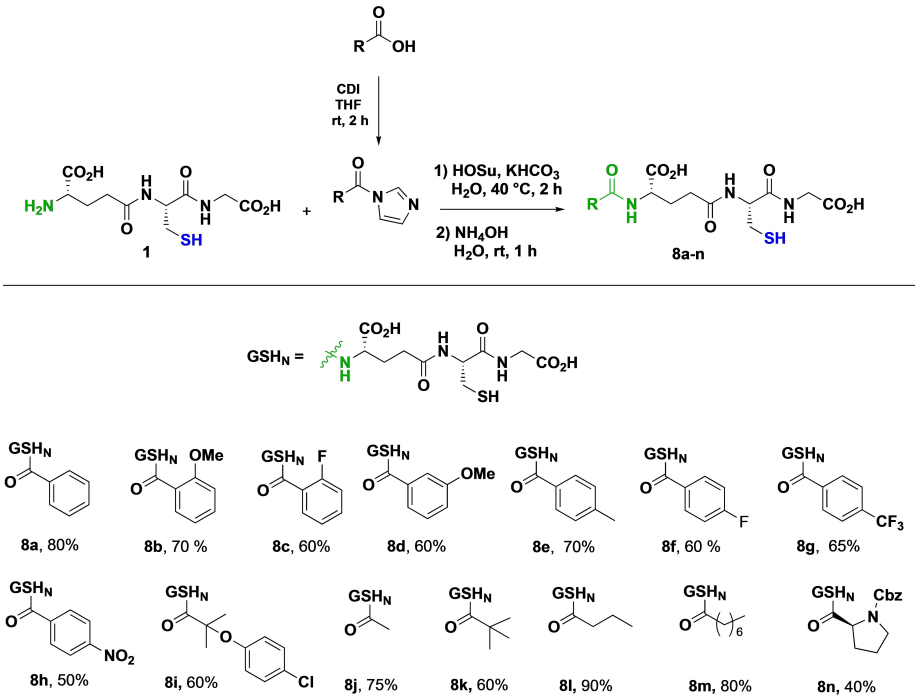
Table 4. Substrate scope investigation of the *N*-acylation of GSH with *N*-acyl imidazole in water.[a]



[a] Unless otherwise noted, all reactions were carried out with GSH (**1**, 307 mg, 1 mmol, 1 equiv) and an *N*-acyl imidazole (2.5 equiv). Isolated yields are reported.

wide applications in both academic and industrial laboratories and should have general applicability for the *S*-acylation and *N*-acylation of biologically active larger peptides and glycopeptides.

Supporting Information

The authors have cited additional references within the Supporting Information.[17,18]

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Conflict of Interests

MR, MM, FB, GP are co-founders of GLUOS, a startup company that develops methods and production based on glutathione modifications.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: acylation · carbonyl imidazole · chemoselectivity · glutathione · water

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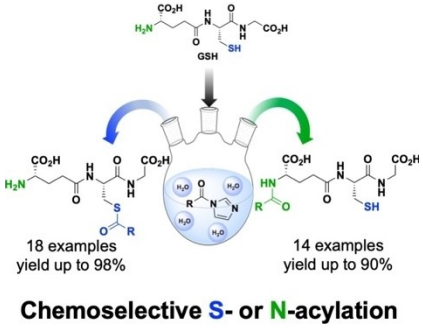
RESEARCH ARTICLE

We report a simple and efficient glutathione acylation reaction for the chemoselective synthesis of *S*- or *N*-acylated glutathione derivatives, under mild conditions and using water as the solvent, by means of the *in-situ* formation of acyl imidazole electrophiles from the respective abundant carboxylic acid.



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Practical and Selective Syntheses of *S*-Acyl and *N*-Acyl Glutathiones with *N*-Acyl Imidazoles in Water