Optimized Diazo Scaffold for Protein Esterification

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S *Supporting Information*

ABSTRACT: The *O*-alkylation of carboxylic acids with diazo compounds provides a means to esterify carboxylic acids in aqueous solution. A Hammett analysis of the reactivity of diazo compounds derived from phenylglycinamide revealed that the (*p*-methylphenyl)-glycinamide scaffold has an especially high reaction rate and ester/alcohol product ratio and esterifies protein carboxyl groups more efficiently than any known reagent.
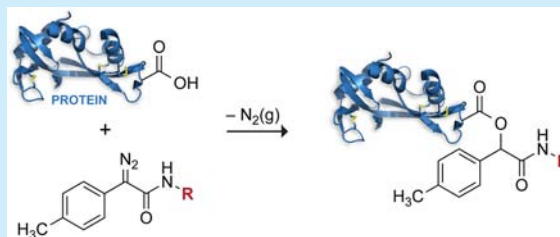
Broad reactivity has made diazo compounds one of the most versatile functional groups in synthetic organic chemistry.[1] Recently, this broad utility has been expanded into the field of chemical biology. For example, the diazo group has been shown to undergo 1,3-dipolar cycloadditions with strained alkynes in a tunable manner. The rates can greatly exceed those of the analogous azide,[2] and the reactions are chemoselective in the presence of mammalian cells.[3] In addition, diazo compounds have been used to label proteins via C−H, N−H, and S−H insertion reactions.[4]

Diazo compounds have another well-known mode of reactivity: esterification of carboxylic acids. We realized that this reactivity could provide unique opportunities in chemical biology. For example, unlike the alkylation of other functional groups, *O*-alkylation of a carboxyl group is bioreversible because mammalian cells contain nonspecific esterases.[5] The esterification of carboxyl groups in proteins and other biomolecules is, however, difficult to effect, as solvent water competes effectively with alcohols for eletrophilic acyl groups. In contrast, esterification reactions mediated by diazo groups rely on the carboxyl group serving as a nucleophile (Scheme 1).[6]

The use of diazo compounds to label proteins was attempted 60 years ago.[7] These initial results were not compelling. A large molar excess (up to 10³-fold) of diazo compound was required to overcome hydrolytic decomposition. Moreover, the reaction was not chemoselective, as amino, sulfhydryl, and phenolic side chains suffered alkylation. Such modifications are potentially deleterious to protein function and not bioreversible.[8]

Previous work in our laboratory suggested that the obstacles in reactivity can be overcome by tuning the reactivity of a diazo group. In particular, we found that the basicity of 9-diazofluorene endows this diazo compound with the ability to label a protein in an aqueous environment.[9] The fluorenyl scaffold is, however, unduly large and not readily amenable to synthetic modification, and its reaction rate and chemo-selectivity are not necessarily maximal.

Accordingly, we sought a scaffold that is optimal for the esterification of carboxyl groups in an aqueous environment. Toward that end, we have examined derivatives of phenyl-glycinamide (Figure 1A). This scaffold delocalizes the electron density on C[α] into an amidic carbonyl group as well as a phenyl group that enables a Hammett analysis[12] of the esterification reaction. Moreover, the amide linkage enables facile installation of useful moieties.

Diazo compounds **1−6** were accessed from derivatives of phenylacetic acid (Figure 1B). Briefly, an azide was installed at the benzylic position of the acid either through displacement of a bromide or by diazo transfer to an existing amine. The ensuing α-azido acids were then coupled to benzylamine and converted to the diazo compound by deimidogenation using a phosphinoester.[10]

In initial experiments, we probed the effect of electron distribution on the reactivity of diazo groups by measuring the rate of esterification in acetonitrile. To do so, we reacted diazo compounds **1−6** with BocGlyOH and measured the second-order rate constants with ^{1}H NMR spectroscopy (Figure S1, Supporting Information). The effect of electron distribution on the reaction rate was dramatic: rate constants spanned over 2 orders of magnitude and increased with the electron-donating
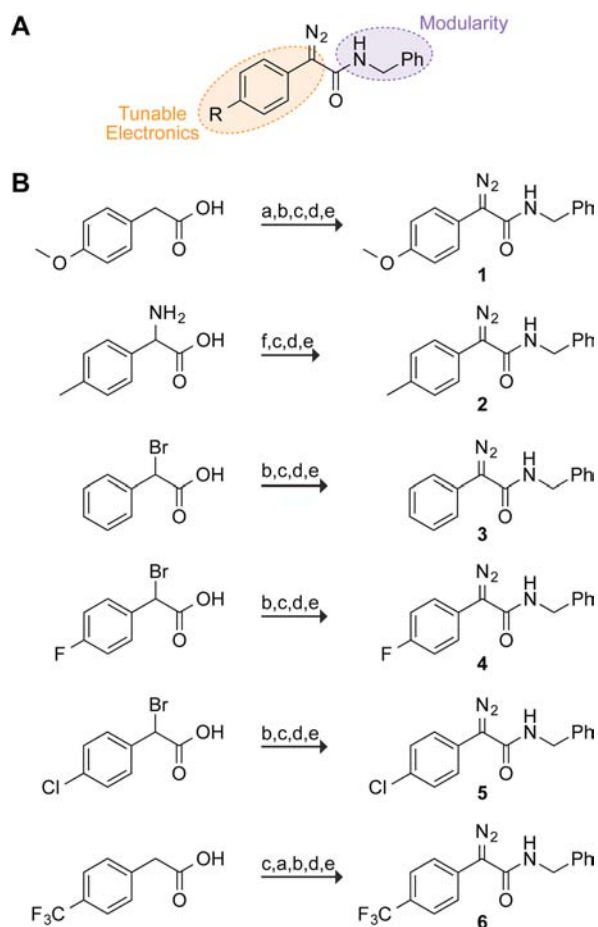
Scheme 1
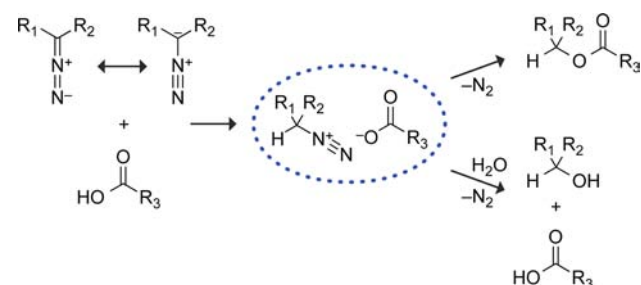
Figure 1. (A) Scaffold for testing the reactivity and selectivity of diazo compounds. (B) Synthetic route to diazo compounds **1−6**. Steps: (a) NBS, AIBN; (b) NaN$_3$, THF/H$_2$O; (c) NHS, DCC, THF; (d) PhCH$_2$NH$_2$, DCM; (e) N-succinimidyl 3-(diphenylphosphino)-propionate, then NaHCO$_3$ or DBU;[10] (f) imidazole-1-sulfonyl azide hydrochloride, DBU, CuSO$_4$, MeOH.[11]
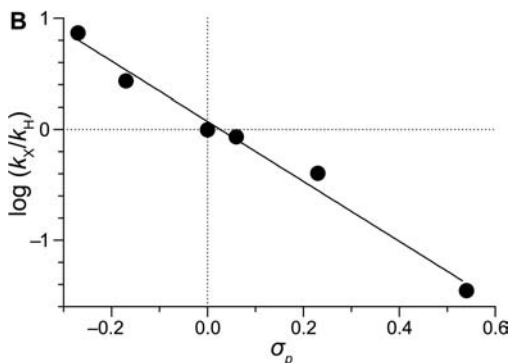

Figure 2. (A) Second-order rate constants for the esterification of BocGlyOH by diazo compounds **1−6** in CD$_3$CN. (B) Hammett plot of the data in panel A. Values of σ_p are from ref 13. $\rho = -2.7$.
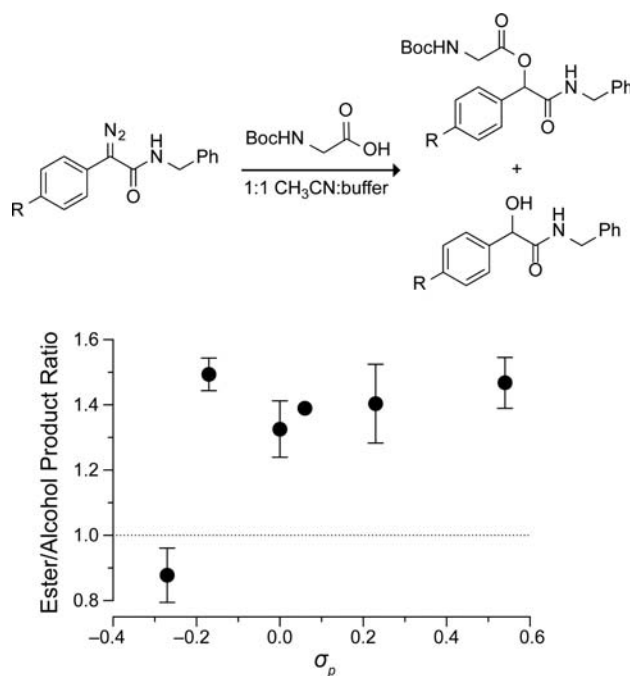

Figure 3. Effect of σ_p value on the chemoselectivity of diazo compounds **1−6** in aqueous solution.
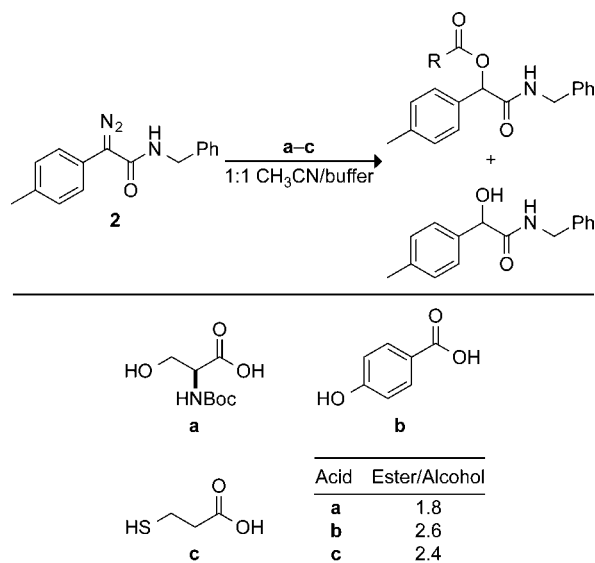

Figure 4. Chemoselectivity of esterification reactions in aqueous solution.

character of the phenyl substituents (Figure 2A). Hammett analysis of these rate constants gave a slope of $\rho = -2.7$ (Figure 2B). This value, which is comparable to those for typical S_N1 reactions, indicates that the esterification reaction is highly sensitive to substituents and that substantial positive charge accumulates during its course,[14] as expected from a mechanism involving an intermediate diazonium ion (Scheme 1).[6]

Next, we sought to find the one compound that demonstrates the greatest selectivity for esterification over hydrolysis in an aqueous environment. Toward that end, we reacted diazo compounds **1−6** with equimolar BocGlyOH in a 1:1 mixture of acetonitrile and 2-(N-morpholino)-ethanesulfonic acid (MES)−HCl buffer at pH 5.5, and we
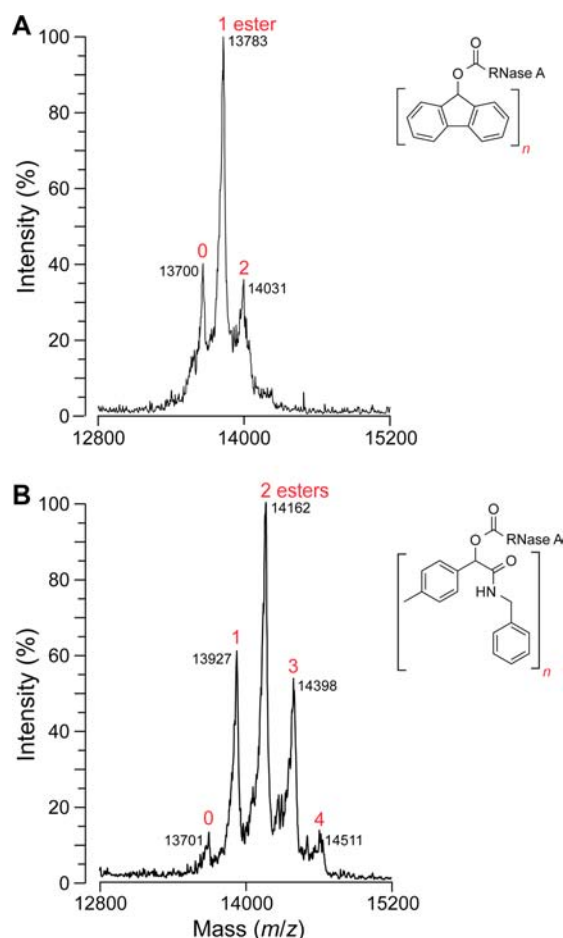
Figure 5. MALDI−TOF mass spectrometry data for esterification of RNase A with (A) 9-diazofluorene and (B) diazo compound **2**.

model protein, ribonuclease A,[16] with 10 equiv of each diazo compound. The reactions were allowed to proceed for 4 h at 37 °C in 1:1 acetonitrile/10 mM MES−HCl buffer at pH 5.5. We then determined the extent of esterification with MALDI−TOF mass spectrometry. We found that diazo compound **2** was approximately 2-fold more efficient than 9-diazofluorene in effecting esterification (Figure 5).

We conclude that diazo compound **2** can be used to esterify proteins in an aqueous environment more efficiently than any other known reagent. Moreover, its modular design enables facile modification with useful moieties. We are now using this diazo compound to attach cell-type targeting, cell-penetration, and pharmacokinetic enhancing modules to proteins of interest.

■ **ASSOCIATED CONTENT**

ⓢ Supporting Information

Experimental procedures for syntheses and kinetic analyses, additional kinetic data, and compound characterization data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b00840.

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Notes

The authors declare no competing financial interest.

determined the ratio of ester-to-alcohol product with ^{1}H NMR spectroscopy.

Surprisingly, the ester/alcohol ratio reached a maximum of 1.4:1 and remained unchanged despite increasing electron withdrawal by the substituents (Figure 3). This result is consistent with a sharp cutoff for the formation of a carboxylate·diazonium intimate ion-pair intermediate that is maintained in a solvent cage by a Coulombic interaction (Scheme 1).[6,15]

On the basis of these experiments, diazo compound **2** was selected for further study, as it demonstrated the fastest rate of those compounds that retain chemoselectivity in an aqueous environment (Figure 4). Because certain diazo compounds undergo O−H and S−H insertion reactions,[1c,4] we sought to ensure that diazo compound **2** would esterify acids selectively in the presence of the sulfhydryl, hydroxyl, or phenolic moieties found on protein side chains. We were gratified to find that diazo compound **2** esterified BocSerOH, *p*-hydroxybenzoic acid, and 3-mercaptopropionic acid in 1:1 acetonitrile/100 mM MES−HCl buffer at pH 5.5 and that no other coupling products were observable by ^{1}H NMR spectroscopy. We also attempted to esterify AlaOH to probe for reaction with an amino group. Consistent with previous observations,[9] diazo compound **2** did not react with either the amino group or the carboxyl group of AlaOH, which was largely zwitterionic in the reaction mixture.

Finally, we compared diazo compound **2** to 9-diazofluorene for the labeling of a protein. To do so, we treated a well-known

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