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Synthesis of Mutual Azo Prodrugs of Anti-inflammatory Agents and Peptides Facilitated by α-Aminoisobutyric Acid

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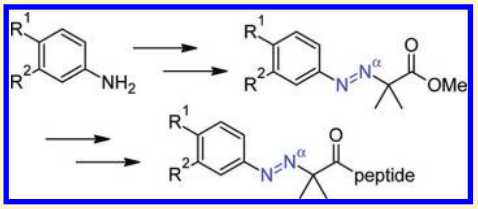
Supporting Information

ABSTRACT: Reported is the synthesis of azo mutual prodrugs of the nonsteroidal anti-inflammatory agents (NSAIDs) 4-aminophenylacetic acid (4-APAA) or 5-aminosalicylic acid (5-ASA) with peptides, including an antibiotic peptide temporin analogue modified at the amino terminal by an α-aminoisobutyric acid (Aib) residue. These prodrugs are designed for colonic delivery of two agents to treat infection and inflammation by the bacterial pathogen *Clostridium difficile*.



INTRODUCTION

The bacterium *Clostridium difficile* can colonize the human colon where it causes diseases such as diarrhea and pseudomembranous colitis, typically associated with disruption of the normal commensal gut microflora by a course of antibiotic treatment.[1] There have been several outbreaks[2] of strains of *C. difficile* that are resistant to many antibiotics of different classes.[3] Recommended routine treatment against infection by *C. difficile* is mainly limited clinically to either metronidazole or the cyclic glycopeptide vancomycin.[4] However, a course of therapy with these antibiotics tends to disturb the gastrointestinal microflora, allowing recolonization by *C. difficile* causing multiple recurrences of infection after treatment.[4] Due to the possibility of emergence of resistance against existing antibiotics, there is a need for the development of new antibiotics for treatment of this infection.

Cationic antimicrobial peptides offer potential to be developed as new antibiotics.[5] Antibiotic peptides are expressed as components of innate immunity in diverse living organisms, including humans,[5] and in the intestines, where they have a role in maintaining the normal gut symbiotic microflora.[6] As a class, antibiotic peptides exhibit potent and broad-spectrum direct microbistatic or microbicidal activity against pathogenic Gram-positive and -negative bacteria; fungi, including yeasts; protozoa; and viruses.[7] Some cationic antimicrobial peptides have selective antitumor and anticancer activity.[8] The antibiotic peptide temporin analogues originally isolated from the skin secretions of the frog *Rana temporaria* are selectively potent against Gram-positive bacteria including clostridia.[9]

C. difficile secretes toxins that cause disease by inflammation of the colonic epithelium.[10] A classical treatment for inflammatory bowel diseases, which include Crohn's disease and ulcerative colitis, is the nonsteroidal anti-inflammatory drug (NSAID) 5-aminosalicylic acid (5-ASA) **1b** which was originally developed as the azo prodrug sulfasalazine.[11,12] Azo prodrugs release therapeutically active amine drugs upon reduction site-specifically by bacterial extracellular azoreductase enzymes and the redox potential in the human colon.[13,14] The azo prodrug APAZA releases the nonsteroidal anti-inflammatory agents 5-aminosalicylic acid and 4-aminophenylacetic acid (4-APAA) **1a**, protecting against damage to the colonic epithelium that is caused by toxin of *C. difficile*.[15]

Our proposed approach to generate new agents targeting infection and disease caused by *C. difficile* is to generate mutual azo prodrugs for concerted site-specific delivery of an antimicrobial peptide and an NSAID (Scheme 1). Protection of the ammonium terminal, which contributes toward the overall positive charge that is important for the activity of an antimicrobial peptide displaying a low net charge, by an azo bond with an anilinic anti-inflammatory agent is employed with the aim of maintaining both components in an inactive state before reaching the colon, thereby avoiding ulceration side effects from the NSAID or disruption of commensal microflora by the antibiotic in the upper gastrointestinal tract.[16−18]

The structural design of the azo mutual prodrugs is shown in Scheme 2. The introduction of an α,α-dialkyl α-amino acid as the N-terminal residue was chosen to avoid an α-proton that could be abstracted allowing tautomerization of the azo to a hydrazone, which could be hydrolyzed *in vivo* and release a toxic hydrazino metabolite. The synthesis results in linkage of the anti-inflammatory agent *via* an azo bond to an α-methylalanine or α-aminoisobutyric acid (Aib) residue. α-Aminoisobutyric acid residues are important constituents toward the activity of peptaibiotics and peptaibols, which are classes of antibiotic peptides of fungal origin.[19,20]

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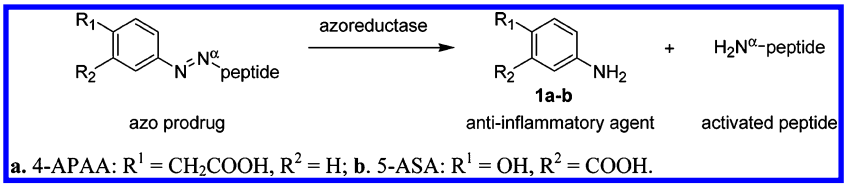
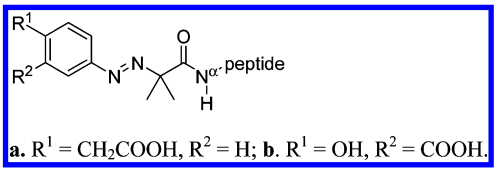
Scheme 1. Activation of Peptide and Anti-inflammatory Agent Mutual Prodrug



a. 4-APAA: $R^1 = CH_2COOH$, $R^2 = H$; **b.** 5-ASA: $R^1 = OH$, $R^2 = COOH$.

Scheme 2. Azo Prodrug Structural Design

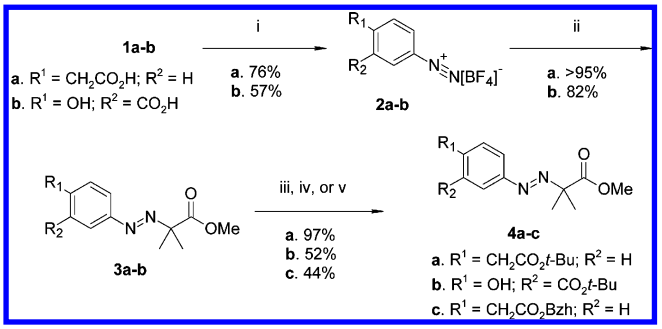


a. $R^1 = CH_2COOH$, $R^2 = H$; **b.** $R^1 = OH$, $R^2 = COOH$.

RESULTS AND DISCUSSION

The complete synthesis of azo mutual prodrugs, as achieved for the peptide temporin with an anti-inflammatory agent, is depicted in Schemes 3 and 7. The azo bond was prepared by

Scheme 3. Preparation of Azo and Protection of Carboxylate[a]



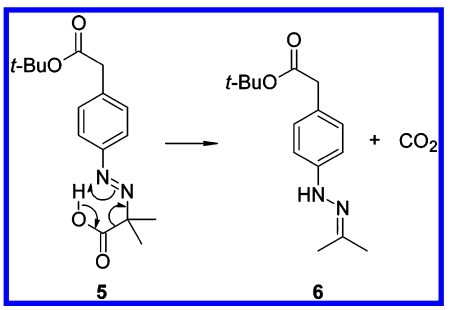
[a]Reagents and conditions: i. 1. HCl, $NaNO_2$. 2. HBF_4. ii. Methyltrimethylsilyl dimethylketene acetal, THF, CH_3CN. iii. Boc_2O, *t*-BuOH, DMAP (cat.). iv. *t*-BuOH, H_2SO_4, $MgSO_4$, CH_2Cl_2. v. Diphenyldiazomethane, 1,4-dioxane, H_2O.

aliphatic diazonium coupling of a silyl ketene acetal to a diazonium fluoborate salt prepared by diazotization of the anilinic[21,22] anti-inflammatory agent starting material. The particular case of diazonium fluoborate **2** was obtainable from the reaction solution of the diazonium chloride by precipitation using fluoboric acid[23] but not sodium fluoborate,[24] despite sodium fluoborate rather than fluoboric acid being recommended in general for obtaining increased yields of diazonium precipitate.[25] The yield for the preparation of the 5-azosalicylic acid **3b** was lower than for the 4-azophenylacetic acid **3a** (Scheme 3).

In the next step, acid-labile protecting groups, benzhydryl and *tert*-butyl esters, were chosen for the carboxyl groups, to allow their deprotection concomitantly to protecting groups of side-chains of amino acid residues. *tert*-Butyl ester as a protecting group was chosen also for its bulkiness, to favor selective deprotection of the methyl ester by alkaline hydrolysis (*vide infra*). Benzhydryl esterification was performed with diphenyldiazomethane,[26] with reaction rate enhanced by a protic mixture of water in 1,4-dioxane solvent, or alternatively, as in the case of preparation of benzhydryl ester **7** (Scheme 5), sterically hindered *tert*-butyl alcohol in dimethylformamide solvent. *tert*-Butyl ester **4a** was prepared by reaction with di-*tert*-butyldicarbonate catalyzed by 4-(dimethylamino)pyridine.[27] Esterification by *tert*-butyl alcohol catalyzed by sulfuric acid with magnesium sulfate desiccant in dichloromethane[28] gave the product **4b** satisfactorily, although the phenolic position is not *tert*-butylated by this method (Table 1).

Attempted deprotection of the methyl ester of **4c** by base hydrolysis using sodium hydroxide was not selective over the benzhydryl ester. Lithium hydroxide in a mixture of water and tetrahydrofuran deprotected the methyl ester of **4a** selectively in the presence of the *tert*-butyl ester. However, the resulting α-azo carboxylate **5** decomposes by decarboxylation, expected to be facilitated by the basicity and mesomeric electronic withdrawal of the azo and the Thorpe–Ingold geminal dialkyl effect (Scheme 4).

Scheme 4. Decarboxylation



Nevertheless, α-azo carboxylic acids have been reported.[29] The similar α-dimethyl carboxylate **8** (Scheme 5) that has an amide instead of α-azo, synthesized in parallel for the

Table 1. Esterification of 3

		3			4		
entry	compound	R^1	R^2	reagents[a]	R^1	R^2	yield (%)
I	**a**	CH_2CO_2H	H	iii	CH_2CO_2t-Bu	H	97
II	**b**	OH	CO_2H	iii	OH	CO_2t-Bu	5
III	**b**	OH	CO_2H	iv	OH	CO_2t-Bu	52
IV	**c**	CH_2CO_2H	H	v	CH_2CO_2Bzh	H	44

[a]Reagents and conditions: iii. Boc_2O, *t*-BuOH, DMAP (10 mol %). iv. *t*-BuOH, H_2SO_4, $MgSO_4$, CH_2Cl_2. v. diphenyldiazomethane, 1,4-dioxane, H_2O.

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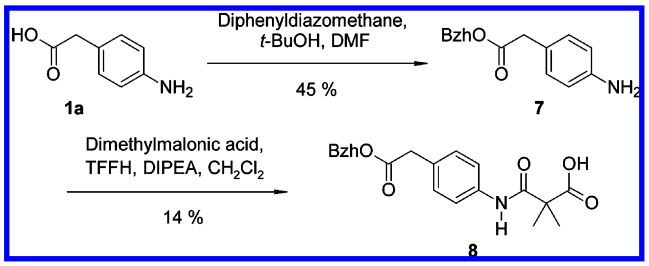
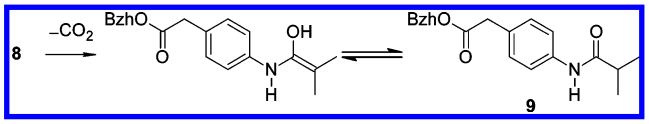
Scheme 5. Amide Isostere



preparation of a negative control, also decarboxylated (Scheme 6) as a side reaction.

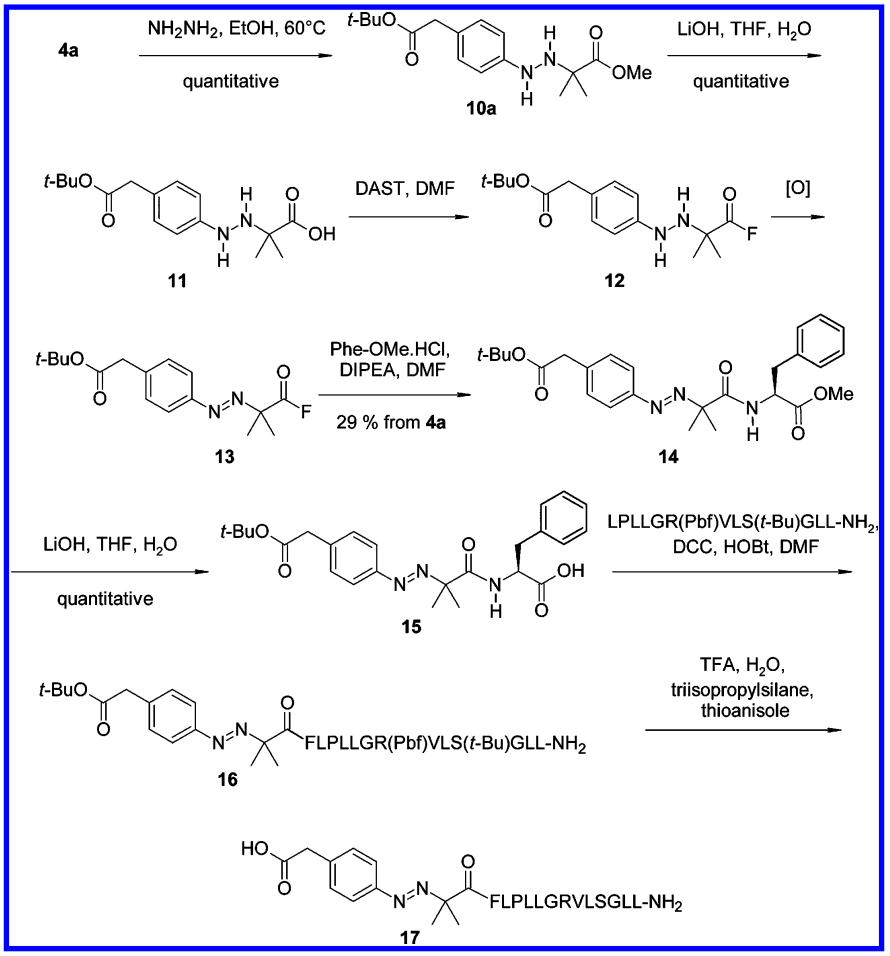
Scheme 6. Decarboxylation



Decarboxylation was circumvented by functional group interconversion, by reduction of the azo **4a** to hydrazo **10a** by hydrazine hydrate[30] (Scheme 7). Yields for reduction of the 4-azophenylacetate **4a** were quantitative. In contrast, reduction of the 5-azosalicylate **4b** by hydrazine could not in this case be used to continue the synthesis as no necessary solid as an isolable precipitate was given from the reaction. (Extraction using vacuum apparatus for deoxygenated conditions was not feasible for the reaction mixture containing solvent quantities of volatile hydrazine.) In any case, redox potential of the azo bond from electronic donation by the phenolic substituent tends to affect the ease of reduction or the propensity for reversion of the hydrazo product to the azo such as upon exposure to oxygen. For example, electron-donating substituents decrease the ease of reduction of aryl azo compounds by clostridial azoreductase.[31] Upon exposure to ordinary atmosphere, the α-hydrazo carboxylate product **11** is susceptible to facile oxidative reversion to azo **5** followed by decarboxylative degradation, thus necessitating deoxygenated reaction and extraction conditions.

The α-hydrazo carboxylic acid **11** gave very poor yields when coupled to an amino acid by amide bond-forming reagents, including HATU, DMTMM,[32] PyBroP,[33] cyanuric fluoride,[34] and TFFH (fluoro-*N*,*N*,*N*′,*N*′-tetramethylformamidinium hexafluorophosphate),[35] typically used for difficult couplings in peptide synthesis. Although hindrance by the α-methyl groups is an effect widely acknowledged in the difficult coupling of the amino acid α-methylalanine, other considerations here include deactivation of the carbonyl by the α-nucleophile effect and electron donation from the α-hydrazo group, or potential reaction of the activated carboxyl group with the hydrazo group. Evidence for the latter contributions was the need to oxidize the α-hydrazo acid fluoride **12** by exposure to atmosphere to convert to the α-azo acid fluoride **13** before

Scheme 7. Completion of the Synthesis of Mutual Prodrug



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coupling to the amino acid. Thus, probably because of the α-hydrazo group, one-pot solution-phase coupling of carboxylic acid **11** to an amino acid *via* the fluorination reagent TFFH was not successful, despite TFFH being effective for coupling of the similarly hindered amino acid α-methylalanine to peptides[35,36] and dimethylmalonic acid to **7** (Scheme 5). The properties of acid fluorides in being more reactive toward nitrogen nucleophiles than oxygen nucleophiles such as water and stable enough to isolate[34] were ideal in these circumstances in which it was necessary to expose the acid fluoride to atmosphere in order to oxidize the hydrazo to azo to allow coupling. The crude acid fluoride **13**, prepared using the fluorinating reagent diethylaminosulfur trifluoride (DAST),[37] was coupled to amino acids without purification.

After coupling to amino acid methyl ester, the methyl ester **14** was deprotected by base hydrolysis before coupling to a protected peptide sequence by a solution-phase convergent condensation using carbodiimide coupling chemistry. The protected peptide was prepared by solid-phase synthesis and cleaved from the resin under mild acidic conditions.[38] The crude condensation product **16** was deprotected without isolation or purification to obtain the final product **17**, which was purified by reverse-phase HPLC.

CONCLUSION

Although azo prodrugs of anti-inflammatory agents and amino acids have been previously reported for site-specific delivery to the colon,[39,40] to the best of our knowledge, here is presented the first preparation of mutual prodrug candidates containing antimicrobial peptides and nonsteroidal anti-inflammatory agents for colonic delivery. The synthesis of an antibacterial peptide temporin A analogue L512TA[41] linked to the anti-inflammatory agent 4-aminophenylacetic acid has been achieved by a strategy that employs an α-methylalanine linker connected to the anti-inflammatory moiety *via* an azo bond involving the α-amino group of the methylalanine residue. Crystal structures have been obtained for the first four of the intermediates in the synthesis, compounds **2a**, **3a**, **4a**, **10a**. All steps, except coupling to the hindered α-dimethyl carboxylic acid, gave good to excellent yields, and the isolated intermediates are obtained in good purity, in most cases accompanied by crystallization directly from the workup or upon standing after extraction. Only one chromatography step is necessary before coupling to the peptide. This synthetic strategy can be applied to the preparation of azo mutual prodrugs of anti-inflammatory agents and peptides. The azo conjugate of 5-aminosalicylic acid and α-methylalanine methyl ester has, for example, been successfully prepared by this methodology for N-terminal modification of antimicrobial peptide candidates.

EXPERIMENTAL SECTION

General. Reverse-phase HPLC chromatography employed Phenomenex Gemini and Varian 5 μm, C-18, 110 Å, 4.6 mm × 250 mm analytical columns; Phenomenex Jupiter 5 μm, C-5, 110 Å, 4.6 mm × 250 mm analytical column; Phenomenex Gemini 5 μm, C-18, 110 Å, 250 mm × 10 mm and Phenomenex Jupiter 15 μm, C-5, 300 Å, 250 mm × 10 mm semi-preparative columns. Crystal structures were determined using data collected with Mo Kα radiation at T = 90 K on a Nonius KappaCCD diffractometer. NMR chemical shifts are not corrected. NMR peaks assignments were assisted by CH COSY.

4-(Carboxymethyl)benzenediazonium Tetrafluoroborate (2a). 4-aminophenylacetic acid **1a** (7.5 g, 50 mmol, 1 equiv) was suspended in concentrated aqueous HCl (20 mL). The mixture was cooled to 0 °C, and a cooled solution of $NaNO_2$ (3.5 g, 50 mmol, 1 equiv) in deionized H_2O (17.5 mL) was added in small portions during 20 min such that the reaction temperature did not exceed 5 °C. The reaction was stirred for a further 30 min until HBF_4 (∼8 M, 17 mL) was added. The resulting precipitate was collected by filtration and sucked dry to obtain a pale solid with discernible needle crystals (11.3 g, 45 mmol, 91% (this yield was increased to 97% when the reaction was allowed for ∼2 h)) which were recrystallized by dissolving in CH_3CN, filtering to remove insoluble solid, precipitation of crystals with Et_2O, and chilling to obtain a pale-pink solid (76%). (Diazonium salts in general are reactive and tend to be unstable in solution above about 5 °C. Care should be taken to maintain all solutions at low temperatures and avoid contact with skin by wearing thick gloves when handling the salts.) Mp 118 °C (dec.); Crystal structure; ^{1}H NMR (CD_3CN, 400 MHz, δ): 9.46 (br s, 1H, COOH), 8.46 (d, J = 8.93 Hz, 2H, aryl CH), 7.86 (d, J = 7.14 Hz, 2H, aryl CH), 3.97 (s, 2H, benzylic CH_2); ^{13}C NMR (CD_3CN, 100.6 MHz, δ): 169.5 (COOH), 150.4 (aryl C), 132.8 (aryl CH), 132.0 (aryl CH), 112.2 (aryl C), 40.0 (benzylic CH_2).

3-Carboxy-4-hydroxybenzenediazonium Tetrafluoroborate (2b). 5-Aminosalicylic acid (5.0 g, 0.03 mol, 1 equiv) was suspended in deionized H_2O (17 mL) and acidified with 37% HCl aqueous solution (13 mL). The mixture was cooled to 0 °C. A solution of $NaNO_2$ (2.3 g, 0.03 mol, 1 equiv) in deionized H_2O (16 mL) was added portionwise. The mixture was stirred for 1 h 30 min. HBF_4 aqueous solution (8 M, 18 mL) was added and stirred before being allowed to stand. The precipitate was collected by filtration to obtain 7.0 g of crystalline solid. The crude solid was treated with CH_3CN and filtered. Solvent was evaporated from the filtrate, and the remaining solid was triturated with Et_2O and collected by filtration. Purified product yield: 4.7 g, 18.6 mmol, 57%, as white solid. ^{1}H NMR (CD_3CN, 400 MHz, δ): 8.75 (d, J = 2.80 Hz, 1H, CH), 8.20 (dd, J_1 = 9.20 Hz, J_2 = 2.80 Hz, 1H, CH), 7.15 (d, J = 9.20 Hz, 1H, CH); ^{13}C NMR (CD_3CN, 100.6 MHz, δ): 171.6 (quaternary C), 168.3 (quaternary C), 138.1 (CH), 138.0 (CH), 121.7 (CH), 115.6 (quaternary C), 101.8 (quaternary C).

(4-{[2-(Methoxycarbonyl)propan-2-yl]diazenyl}phenyl)acetic Acid (3a). To a solution of diazonium tetrafluoborate **2a** (5.0 g, 20 mmol, 1.0 equiv) in anhydrous THF (23.3 mL) cooled to −5 °C under N_2 gas in an overdried flask was added methyltrimethylsilyl dimethylketene acetal (5.8 mL, 28.6 mmol, 1.43 equiv) slowly in 1 mL portions *via* syringe. Anhydrous CH_3CN (23.3 mL) was added and the mixture was stirred for two hours. The mixture was concentrated by rotary evaporation without heat and with the flask covered by aluminum foil. The residue was treated with H_2O and extracted with $CHCl_3$. The combined $CHCl_3$ extracts were washed with H_2O, dried over anhydrous $MgSO_4$, and filtered, and solvent was evaporated to obtain an oil that crystallized upon standing (5.6 g, 21.4 mmol, yield: quantitative). Crystal structure. IR (KBr disk) 3449, 2992, 2954, 2731, 2650, 2556, 2361, 2341, 1738, 1713, 1608, 1523, 1495, 1463, 1433, 1408, 1363, 1336, 1287, 1238, 1200, 1155, 1105, 1014, 994, 934, 846, 819, 797, 758, 732, 680, 508 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz, δ): 8.80 (br s, 1H, COOH), 7.59 (d, J = 8.40 Hz, 2H, CH), 7.29 (d, J = 8.40 Hz, 2H, CH), 3.67 (s, 3H, CO_2CH_3), 3.61 (s, 2H, CH_2), 1.51 (s, 6H, CH_3); ^{13}C NMR ($CDCl_3$, 100.6 MHz, δ): 176.5 (CO_2Me), 174.1 (CO_2H), 150.8 (aromatic C), 136.4 (aromatic C), 130.1 (CH), 122.7 (CH), 75.6 (CN═N), 52.3 (CH_3), 40.8 (CH_2), 23.2 (CH_3); λ_{max} (CH_3CN) $\varepsilon_{296.6\ nm}$: 2887 L mol^{-1} cm^{-1}, $\varepsilon_{402.2\ nm}$: 274 L mol^{-1} cm^{-1}; *Anal.* Calcd for $C_{13}H_{16}N_2O_4$: C, 59.08; H, 6.10; N, 10.60. Found: C, 59.22; H, 6.15; N, 10.38.

2-Hydroxy-5-{[2-(methoxycarbonyl)propan-2-yl]diazenyl}-benzoic Acid (3b). Diazonium tetrafluoborate **2b** (3.0 g, 12 mmol, 1 equiv) was dissolved in anhydrous THF (40 mL) and anhydrous CH_3CN (60 mL) under N_2 gas. Methyl trimethylsilyl dimethylketene acetal (5 mL, 25 mmol, 2 equiv) was delivered *via* syringe. The reaction was performed at −5 °C for 3 h 20 min. Solvent was evaporated. The residual solid was treated with H_2O and extracted with $CHCl_3$. The chlorinated phase was washed with H_2O, and the aqueous washings were acidified with concentrated 37% HCl solution (20 mL) and extracted with $CHCl_3$. The combined chlorinated

extracts were washed with H_2O, and solvent was evaporated to obtain a brown solid (2.6 g, 9.8 mmol, 82%). IR (KBr disk) 2992, 2592, 1737, 1671, 1610, 1585, 1519, 1509, 1483, 1467, 1437, 1378, 1360, 1330, 1277, 1250, 1190, 1143, 1073, 1004, 974, 899, 844, 831, 802, 791, 773, 717, 703, 679, 638, 592, 571, 553, 512 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz, δ): 10.78 (s, 1H, OH), 8.26 (d, J = 2.48 Hz, 1H, CH), 7.85 (dd, J_1 = 8.93 Hz, J_2 = 2.48 Hz, 1H, CH), 6.99 (d, J = 8.93 Hz, 1H, CH), 3.70 (s, 3H, CO_2CH_3), 1.52 (s, 6H, CH_3); ^{13}C NMR ($CDCl_3$, 100.6 MHz, δ): 174.6 (COOH), 173.5 (CO_2Me), 164.2 (aryl COH), 144.2 (aryl C), 129.6 (aryl CH), 127.0 (aryl CH), 118.5 (aryl CH), 111.3 (aryl C), 75.3 (CN═N), 52.5 (CO_2CH_3), 23.3 (CH_3); λ_{max} (CH_3CN) $\varepsilon_{315.2\ nm}$: 2743 L mol^{-1} cm^{-1}, $\varepsilon_{395.6\ nm}$: 193 L mol^{-1} cm^{-1}; ES^--MS 265 $[M - 1]^+$; HRMS ES^+ TOF Calcd for $C_{12}H_{14}N_2O_5Na$: 289.0800. Found: 289.0789.

Methyl 2-({4-[(*tert*-Butoxycarbonyl)methyl]phenyl}-diazenyl)-2-methylpropanoate (4a). Carboxylic acid **3a** (2.2 g, 8.3 mmol, 1 equiv) was dissolved in *t*-BuOH (25 mL) at 26 °C, followed by di-*tert*-butyl dicarbonate (2.5 g, 11.6 mmol, 1.4 equiv) and 4-(dimethylamino)pyridine (0.1 g, 0.87 mmol, 0.1 equiv). The mixture was stirred for 2 h and 21 min. The mixture was concentrated by rotary evaporation, extracted with CH_2Cl_2, and washed with aqueous HCl solution and H_2O. The chlorinated extract was dried over anhydrous $MgSO_4$ powder and filtered, and solvent was removed by rotary evaporation. The product crystallized upon standing after evaporation of solvent. Product is a yellow solid (2.6 g, 8 mmol, 97%). Crystal structure; mp 60 °C; IR (KBr disk) 3447, 2983, 2934, 1740, 1608, 1518, 1457, 1435, 1420, 1394, 1371, 1341, 1278, 1232, 1188, 1147, 1011, 986, 942, 882, 848, 799, 757, 694, 578, 511 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz, δ): 7.58 (d, J = 8.40 Hz, 2H, CH), 7.29 (d, J = 8.40 Hz, 2H, CH), 3.68 (s, 3H, CO_2CH_3), 3.50 (s, 2H, benzylic CH_2), 1.52 (s, 6H, CH_3), 1.36 (s, 9H, $C(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 100.6 MHz, δ): 174.0 (CO_2Me), 170.4 (CO_2t-Bu), 150.6 (aromatic C), 137.7 (aromatic C), 129.8 (aromatic CH), 122.5 (aromatic CH), 81.1 (quaternary $C(CH_3)_3$), 75.5 (quaternary CN═N), 52.2 (CH_3), 42.56 (CH_2), 28.0 (CH_3), 23.2 (CH_3); λ_{max} (CH_3CN) $\varepsilon_{290.6\ nm}$: 3801 L mol^{-1} cm^{-1}, $\varepsilon_{401.0\ nm}$: 118 L mol^{-1} cm^{-1}; *Anal.* Calcd for $C_{17}H_{24}N_2O_4$: C, 63.73; H, 7.55; N, 8.74. Found: C, 63.77; H, 7.64; N, 8.42.

***tert*-Butyl 2-Hydroxy-5-{[2-(methoxycarbonyl)propan-2-yl]-diazenyl}benzoate (4b, Table 1, entry III).** Anhydrous $MgSO_4$ powder (1.2 g, 10 mmol, 4 equiv) was stirred with concentrated H_2SO_4 (0.14 mL, 1.3 mmol, 5 equiv) in CH_2Cl_2 for 15 min. Carboxylic acid **3b** (0.7 g, 2.6 mmol, 1 equiv) was added, followed by *t*-BuOH (1.2 mL, 13 mmol, 5 equiv). A stopper was placed over the neck of the flask, and the mixture was stirred for 17 h 20 min. The reaction was quenched by stirring with saturated $NaHCO_3$ aqueous solution (19.3 mL). The separated aqueous phase was extracted with CH_2Cl_2. The chlorinated phase was washed with brine (15 + 20 mL) and H_2O (2 × 20 mL), separated, dried over anhydrous $MgSO_4$ powder, and filtered. The solvent was evaporated from the filtrate to obtain a brown crystalline solid. Yield: 0.4 g, 1.4 mmol, 52%. Crystal structure; mp 76 °C; IR (KBr disk) 3449, 3125, 2980, 2926, 2853, 1738, 1672, 1616, 1584, 1519, 1474, 1432, 1374, 1356, 1296, 1250, 1222, 1154, 1079, 1014, 992, 911, 847, 799, 758, 746, 719, 699, 569, 544, 518 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz, δ): 11.35 (s, 1H, OH), 8.12 (d, J = 2.50 Hz, 1H, aryl CH), 7.74 (dd, J_1 = 8.92 Hz, J_2 = 2.50 Hz, 1H, aryl CH), 6.93 (d, J = 8.92 Hz, 1H, aryl CH), 3.69 (s, 3H, CO_2CH_3), 1.57 (s, 9H, $(CH_3)_3$), 1.52 (s, 6H, CH_3); ^{13}C NMR ($CDCl_3$, 100.6 MHz, δ): 174.1 (CO_2Me), 169.6 (CO_2t-Bu), 164.1 (aryl COH), 144.1 (aryl C), 127.8 (aryl CH), 126.7 (aryl CH), 118.3 (aryl CH), 113.6 (aryl C), 83.7 (quaternary C, $C(Me)_3$); 75.1 (CN═N), 52.3 (CO_2CH_3), 28.2 (CH_3, $C(CH_3)_3$); 23.3 (CH_3); λ_{max} (CH_3CN) $\varepsilon_{297.2\ nm}$: 6266 L mol^{-1} cm^{-1}, $\varepsilon_{393.2\ nm}$: 169 L mol^{-1} cm^{-1}; HRMS ES^- TOF Calcd for $C_{16}H_{21}N_2O_5$: 321.1450. Found: 321.1447.

Methyl 2-({4-[(Diphenylmethoxycarbonyl)methyl]phenyl}-diazenyl)-2-methylpropanoate (4c, Table 1, Entry IV). Carboxylic acid **3a** (0.6 g, 2.16 mmol, 1 equiv) was placed in a 50-mL round-bottom flask with deionized H_2O (4 mL). Diphenyldiazomethane as a solution in 1,4-dioxane (4 mL) was added, and the mixture of phases was stirred vigorously at ambient temperature for 1 day. Solvent was removed by rotary evaporation. The resulting oily residue was purified by chromatography on silica gel using petroleum ether with increasing proportion of CH_2Cl_2 as mobile phase. After evaporation of solvent, product was obtained as a partially solidified oil (0.4 g, 0.94 mmol, 44%). ^1H NMR ($CDCl_3$, 400 MHz, δ): 7.56 (d, 2H, CH), 7.3 (d, 2H, CH), 7.3–7.1 (overlapping ms, 10H, CH), 6.79 (s, 1H, benzhydrylic CH), 3.68 (s, 2H, CH_2), 3.66 (s, 3H, CO_2CH_3), 1.51 (s, 6H, CH_3); ^{13}C NMR ($CDCl_3$, 100.6 MHz, δ): 174.0 (CO_2Me), 170.0 (CO_2Bzh), 150.8 (aromatic C), 140.0 (benzhydryl C), 136.7 (aromatic C), 130.1 (CH), 128.6 (benzhydryl CH), 128.0 (benzhydryl CH), 127.0 (benzhydryl CH), 122.7 (CH), 77.4 (benzhydrylic CH), 75.6 (CN═N), 52.3 (CH_3), 41.4 (CH_2), 23.2 (CH_3); ES^+-MS 431 $[M + 1]^+$, 453 $[M + Na]^+$; HRMS ES^+ TOF Calcd for $C_{26}H_{26}N_2O_4Na$: 453.1790. Found: 453.1808.

2-({4-[(*tert*-Butoxycarbonyl)methyl]phenyl}diazenyl)-2-methylpropanoic Acid (5). Methyl ester **4a** (121 mg, 0.38 mmol, 1 equiv) was stirred with 1 M LiOH aqueous solution (0.76 mL, 0.76 mmol, 2 equiv) in THF (5.8 mL) and deionized H_2O (2.42 mL) for 1 day. The mixture was treated with 1 M HCl aqueous solution (24 mL) to bring the pH to neutral or slightly acidic. The mixture was extracted with EtOAc (20 mL), and solvent was removed by rotary evaporation. ^1H NMR ($CDCl_3$, 400 MHz, δ): 9.35 (br s, 1H), 7.62 (d, J = 8.40 Hz, 2H, aryl CH), 7.32 (d, J = 8.40 Hz, 2H, aryl CH), 3.52 (s, 2H, benzylic CH_2), 1.51 (s, 6H, CH_3), 1.37 (s, 9H, $(CH_3)_3$).

***tert*-Butyl {4-[2-(Propan-2-ylidene)hydrazinyl]phenyl}-acetate (6).** By decarboxylation of α-azo carboxylic acid **5**. ^1H NMR ($CDCl_3$, 400 MHz, δ): 7.05 (d, J = 8.5, 2H), 6.90 (d, J = 8.5 Hz, 2H), 3.34 (s, 2H), 1.95 (s, 3H), 1.78 (s, 3H), 1.34 (s, 9H); ^{13}C NMR ($CDCl_3$, 100.6 MHz, δ): 170.5, 143.8, 143.1, 128.9, 124, 111.9, 79.4, 41.0, 27.0, 24.2, 14.6; HRMS ES^- TOF Calcd for $C_{15}H_{21}N_2O_2$: 261.1603. Found: 261.1596.

Diphenylmethyl (4-aminophenyl)acetate (7). 4-Aminophenylacetic acid **1a** (1.0 g, 6.6 mmol, 1 equiv) was dissolved in DMF (10 mL) and *t*-BuOH (7 mL). A solution of diphenyldiazomethane (~15 mmol) in DMF (10 mL) was added, and the mixture was stirred at ambient temperature for 19 h 5 min. The mixture was washed with deionized H_2O (3 × 40 mL) and extracted with CH_2Cl_2. The extract was dried over anhydrous $MgSO_4$ powder, and filtered, and solvent was evaporated to leave a deep red-pink liquid. The crude mixture was purified by column chromatography, using petroleum ether and CH_2Cl_2 mixture mobile phase. The product was obtained as a pale solid (0.9 g, 3 mmol, 45%). R_f 0.4–0.3 on silica. ^1H NMR ($CDCl_3$, 400 MHz, δ): 7.26–7.16 (overlapping ms, 10H, benzhydryl aryl CH), 6.99 (d, J = 8.36 Hz, 2H, aryl CH), 6.78 (s, 1H, benzhydrylic CH), 6.57 (d, J = 8.40 Hz, 2H, aryl CH), 3.56 (br s, 2H, NH_2), 3.54 (s, 2H, benzylic CH_2); ^{13}C NMR ($CDCl_3$, 100.6 MHz, δ): 171.1 (CO_2Bzh), 145.4 (aryl C), 138.4 (benzhydryl C), 130.3 (aryl CH), 128.5 (CH), 127.8 (CH), 127.0 (CH), 123.8 (aryl C), 115 (aryl CH), 77 (benzhydrylic CH), 40.8 (benzylic CH_2); ES^+-MS 340 $[M + Na]^+$, 657 $[2M + Na]^+$; HRMS ES^+ TOF Calcd for $C_{21}H_{19}NO_2Na$: 340.1313. Found: 340.1317.

3-({4-[(Diphenylmethoxycarbonyl)methyl]phenyl}amino)-2,2-dimethyl-3-oxopropanoic acid (8). Dimethylmalonic acid (274 mg, 2.08 mmol, 2.2 equiv) and TFFH (550 mg, 2.08 mmol, 2.2 equiv) were dissolved in CH_2Cl_2 (2.9 mL) with *N*,*N*-diisopropylethyl amine (0.7 mL, 4.158 mmol, 4.4 equiv) and added to a solution of diphenylmethyl (4-aminophenyl)acetate **7** (300 mg, 0.945 mmol, 1.0 equiv) in CH_2Cl_2 (2 mL). The mixture was stirred for 19 h 20 min at ambient temperature. Solvent was evaporated. The residue was purified by column chromatography through silica gel in *n*-hexane, using *n*-hexane and dichloromethane mixtures as eluent. The product has higher R_f on silica with dichloromethane mobile phase than the starting material. Product was obtained as a yellow solid (57.7 mg, 0.13 mmol, 14%). ^1H NMR ($CDCl_3$, 400 MHz, δ): 7.72 (d, 2H, aryl CH), 7.27–7.15 (overlapping ms, 12H, aryl CH), 6.79 (s, 1H, benzhydrylic CH), 3.65 (s, 2H, benzylic CH_2), 1.41 (s, 6H, CH_3); ^{13}C NMR ($CDCl_3$, 100.6 MHz, δ): 207.0 (COOH), 172.8 (CONH), 170.0 (CO_2Bzh), 139.9 (benzhydryl C), 132.4 (aryl C), 130.2 (aryl CH), 128.5 (CH), 128.0 (CH), 127.0 (CH), 119.2 (aryl CH), 77.5

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(benzhydrylic CH), 61.8 (quaternary C), 41.2 (benzylic CH_2), 17.8 (CH_3) (one aryl C not assigned); ES^--MS 430 $[M - 1]^-$, 863 $[2M - 1]^-$; HRMS ES^- TOF Calcd for $C_{26}H_{24}NO_5$: 430.1654. Found: 430.1663.

Diphenylmethyl {4-[(2-Methylpropanoyl)amino]phenyl}-acetate (9). Side product by decarboxylation of carboxylic acid **8**, isolated by column chromatography. ^1H NMR ($CDCl_3$, 400 MHz, δ): 7.39 (d, J = 8.40 Hz, 2H, aryl CH), 7.3−7.15 (overlapping ms, 10H, benzhydryl aryl CH), 7.10 (d, J = 8.32 Hz, 2H, aryl CH), 6.77 (s, 1H, benzhydrylic CH), 3.59 (s, 2H, benzylic CH_2), 2.39 (septet, J = 6.80 Hz, 1H, CH), 1.13 (d, J = 6.80 Hz, 6H, CH_3); ^{13}C NMR ($CDCl_3$, 100.6 MHz, δ): 175.2 (CONH), 170.5 (CO_2Bzh), 140.1 (benzhydryl C), 137.2 (aryl C), 129.9 (aryl CH), 128.5 (benzhydryl CH), 127.9 (benzhydryl CH), 127.0 (benzhydryl CH), 119.8 (aryl CH), 77 (benzhydrylic CH), 41.0 (benzylic CH_2), 36.7 (CH), 19.6 (CH_3); ES^--MS 386 $[M - 1]^-$; HRMS ES^+ TOF Calcd for $C_{25}H_{25}NO_3Na$: 410.1732. Found: 410.1738.

Methyl 2-(2-{4-[(*tert*-Butoxycarbonyl)methyl]phenyl}-hydrazinyl)-2-methylpropanoate (10a). Azo **4a** (1.2 g, 3.7 mmol, 1 equiv) was dissolved in EtOH (100 mL) heated to 60 °C. NH_2NH_2 hydrate (20 mL) was added, and the solution was stirred for 2 h. The mixture was poured on ice and concentrated by rotary evaporation until a white precipitate appeared. The white solid was collected by filtration and washed with deionized H_2O. The analytical sample was obtained by recrystallization by dissolving in cold EtOH, adding deionized H_2O, and chilling to −5 °C. White flakes. Yield: quantitative. Crystal structure; mp 79 °C; ^1H NMR ($CDCl_3$, 400 MHz, δ): 7.00 (d, J = 8.40 Hz, 2H, aryl CH), 6.78 (d, J = 8.40 Hz, 2H, aryl CH), 5.45 (br s, 1H, NH), 4.01 (br s, 1H, NH), 3.69 (s, 3H, CO_2CH_3), 3.34 (s, 2H, benzylic CH_2), 1.36 (s, 9H, $C(CH_3)_3$), 1.27 (s, 6H, CH_3); ^{13}C NMR ($CDCl_3$, 100.6 MHz, δ): 176.5 (CO_2Me), 170.6 (CO_2t-Bu), 148.1 (aryl C), 128.6 (aryl CH), 123.6 (aryl C), 111.8 (aryl CH), 79.4 ($C(CH_3)_3$), 61.3 (CNHNH), 51.2 (CO_2CH_3), 40.8 (benzylic CH_2), 27.0 ($C(CH_3)_3$), 22.4 (CH_3); λ_{max} (CH_3CN) $\varepsilon_{295.4\ nm}$: 625 L mol^{-1} cm^{-1}.

2-(2-{4-[(*tert*-Butoxycarbonyl)methyl]phenyl}hydrazinyl)-2-methylpropanoic Acid (11). Methyl ester **10a** (1 equiv) was stirred with LiOH (2 equiv) in deoxygenated deionized H_2O (20 mL per gram of ester) and deoxygenated THF (46.7 mL per gram of ester) for 24 h. Reaction was quenched by 1 M HCl aqueous solution (11.7 mL per gram of carboxylate) and extracted with deoxygenated CH_2Cl_2. Solvent was evaporated to yield a pale solid. Yield: quantitative. ^1H NMR ($CDCl_3$, 400 MHz, δ): 7.02 (d, J = 8.36 Hz, 2H), 6.88 (d, J = 8.32 Hz, 2H), 3.36 (s, 2H), 1.38 (s, 6H), 1.36 (s, 9H); ^{13}C NMR ($CDCl_3$, 100.6 MHz, δ): 181.8, 171.8, 149.0, 129.72, 124.9, 113.0, 80.6, 62.1, 41.8, 28.1, 23.4.

***tert*-Butyl {4-[2-(1-Fluoro-2-methyl-1-oxopropan-2-yl)-hydrazinyl]phenyl}acetate (12).** Carboxylic acid **11** (205.4 mg, 0.67 mmol, 1 equiv) was dissolved in deoxygenated CH_2Cl_2 (2 mL) and cooled to 0 °C. A solution of diethylaminosulfur trifluoride (0.09 mL) in deoxygenated CH_2Cl_2 (1 mL) was added dropwise. Reaction was allowed under N_2 gas for 1 h 10 min. The mixture was diluted with CH_2Cl_2 and washed with H_2O. The extract was dried over anhydrous $MgSO_4$ powder, filtered, and solvent was evaporated to leave a dark-brown residue. ^1H NMR ($CDCl_3$, 400 MHz, δ): 7.01 (d, J = 8.48 Hz, 2H), 6.8 (d, J = 8.52 Hz, 2H), 3.35 (s, 2H), 1.36 (s, 9H) (methyl groups not assigned).

***tert*-Butyl {4-[(1-Fluoro-2-methyl-1-oxopropan-2-yl)-diazenyl]phenyl}acetate (13).** Hydrazo **12** was allowed to oxidize in air. ^1H NMR ($CDCl_3$, 400 MHz, δ): 7.61 (d, J = 8.36 Hz, 2H), 7.31 (d, J = 8.40 Hz, 2H), 3.51 (s, 2H), 1.61 (s, 6H), 1.36 (s, 9H).

***N*$^\alpha$-[*α*-({4-[(*tert*-Butoxycarbonyl)methyl]phenyl}diazenyl)-isobutyryl]-Phe-OMe (14).** Acid fluoride **13** (crude, maximum 0.9 mmol, ~1 equiv) and H-Phe-OMe·HCl (135 mg, 0.63 mmol, 1 equiv) were treated with *N*,*N*-diisopropylethyl amine (0.22 mL, 1.26 mmol, 2 equiv) and dissolved in anhydrous DMF (0.5 mL). The mixture was stirred for 21 h 44 min. The mixture was diluted with CH_2Cl_2 and washed with 1 M HCl aqueous solution (3 × 10 mL) and H_2O (2 × 15 mL). The product was purified by column chromatography through silica. Yield: 12.4 mg, 0.03 mmol, 29% from **4a**. ^1H NMR ($CDCl_3$, 400 MHz, δ): 7.49 (d, J = 8.32 Hz, 2H, aryl CH), 7.48 (m, 1H, NH), 7.30 (d, J = 8.32 Hz, 2H, aryl CH), 7.17−7.12 (overlapping ms, 3H, Phe aryl CH), 7.04−6.99 (m, 2H, Phe aryl CH), 4.95 (dt, J_1 = 7.96 Hz, J_2 = 5.80 Hz, 1H, CH), 3.67(s, 3H, CO_2CH_3), 3.53 (s, 2H, benzylic CH_2), 3.12 (ms, 2H, Phe benzylic CH_2), 1.38 (s, 9H, $(CH_3)_3$), 1.34 (s, 3H, CH_3), 1.31 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100.6 MHz, δ): 174.4 (CONH), 171.9 (CO_2Me), 170.3 (CO_2t-Bu), 150.6 (aryl C), 138.1 (aryl C), 135.8 (aryl C), 130.0 (aryl CH), 129.3 (Phe aryl CH), 128.6 (Phe aryl CH), 127.1 (Phe aryl CH), 122.5 (aryl CH), 81.2 (CMe_3), 74.2 (CN═N), 52.8 (CH), 52.4 (CO_2CH_3), 42.5 (benzylic CH_2), 37.9 (Phe benzylic CH_2), 28.0 ($(CH_3)_3$), 23.4 (CH_3), 23.1 (CH_3); ES^+-MS 468 $[M + 1]^+$, 490 $[M + Na]^+$, 958 $[2M + Na]^+$; HRMS ES^+ TOF Calcd for $C_{26}H_{33}N_3O_5Na$: 490.2318. Found: 490.2314.

***N*$^\alpha$-[*α*-({4-[(*tert*-Butoxycarbonyl)methyl]phenyl}diazenyl)-isobutyryl]-Phe-OH (15).** Methyl ester **14** (66 mg, 0.14 mmol, 1 equiv) was stirred with LiOH (6.2 mg, 0.26 mmol, 1.8 equiv) in H_2O (0.23 mL) and THF (0.54 mL) for 2 h. The mixture was acidified with 1 M HCl aqueous solution (1.14 mL) and extracted with CH_2Cl_2. Solvent was evaporated under vacuum to obtain a yellow microcrystalline solid. Yield: 65 mg, 0.14 mmol, quantitative. Mp 101 °C; ^1H NMR ($CDCl_3$, 400 MHz, δ): 9.04 (br s, 1H, COOH), 7.55 (d, J = 7.68 Hz, 1H, NH), 7.46 (d, J = 8.32 Hz, 2H, aryl CH), 7.29 (d, J = 8.32 Hz, 2H, aryl CH), 7.17−7.12 (overlapping ms, 3H, Phe aryl CH), 7.12−7.05 (m, 2H, Phe aryl CH), 4.95 (m, J_1 = 7.60 Hz, J_2 = 5.9 Hz, 1H, CH), 3.52 (s, 2H, benzylic CH_2), 3.18 (ms, 2H, Phe benzylic CH_2), 1.38 (s, 9H, $CO_2C(CH_3)_3$), 1.33 (s, 3H, CH_3), 1.30 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100.6 MHz, δ): 174.2 (C═O), 173.8 (C═O), 169.4 (CO_2t-Bu), 149.6 (aryl C), 137.1 (aryl C), 134.6 (aryl C), 128.9 (aryl CH), 128.4 (Phe aryl CH), 127.6 (Phe aryl CH), 126.2 (Phe aryl CH), 121.5 (aryl CH), 80.3 (CMe_3), 73.0 (CN═N), 51.9 (CH), 41.5 (benzylic CH_2), 36.2 (Phe benzylic CH_2), 27.0 ($(CH_3)_3$), 22.3 (CH_3), 22.0 (CH_3); ES^--MS 452 $[M - 1]^-$, 906 $[2M - 1]^-$; HRMS ES^+ TOF Calcd for $C_{25}H_{32}N_3O_5$: 454.2342. Found: 454.2352.

General Procedure for Peptide Synthesis. *Solid-Phase Synthesis.* Peptides were synthesized using automated peptide synthesizer using Fmoc N^α-protection strategy on a Rink amide MBHA resin solid support (typical substitution: 0.7 mmol/g). Protected peptides for solution phase were synthesized on a Sieber amide resin.

Cleavage and Deprotection. Deprotection of amino acid residue side groups and cleavage of peptide from resin was achieved by stirring with a mixture of TFA (6 mL), deionized H_2O (450 μL), triisopropylsilane (450 μL), and thioanisole (900 μL). If peptide contained tryptophan, methionine, or cysteine, 1,2-ethanedithiol (450 μL) was also added to the cleavage mixture. Stirring was allowed for 2 h plus an additional 30 min for each arginine residue up to a maximum of 4 h. The resin was then removed by filtration over sintered glass or frit. The peptide was precipitated and washed by the addition of Et_2O to the filtrate and collecting as a pellet by centrifugation at 2.8 × 1000 rpm. The pellet was dissolved in H_2O, with CH_3CN if necessary, and lyophilized.

Purification. Crude peptides were purified by semipreparative reverse-phase HPLC. Unless otherwise stated, purification employed a Phenomenex Gemini 5 μm, C-18, 110 Å, 250 mm × 10 mm and elution involved a 30 min gradient of H_2O (0.1% TFA)/CH_3CN (0.1% TFA) 95:5−35:65, followed by isocratic elution.

Analysis. Purity and retention time were assessed by reverse-phase HPLC with Varian 5 μm, C-18, 110 Å, 4.6 mm × 250 mm analytical column, with a 30 min elution gradient of H_2O (0.1% TFA)/CH_3CN (0.1% TFA) 95:5−35:65, followed by isocratic elution. Identity of each peptide was confirmed by MALDI-MS or $ES^\pm$-MS. Absorbance maxima were determined by photodiode array detector.

General Procedure for Preparation of Protected Peptide. Protected peptide on Sieber amide resin was placed over a frit filter and rinsed with a solution of 1% v/v TFA in CH_2Cl_2, with the filtrate being collected in a 12% v/v solution of *N*,*N*-diisopropylethyl amine in MeOH. The filtrate solvent was evaporated, and the remaining liquid was diluted with $CHCl_3$ and washed with deionized H_2O. The solvent was removed from the chlorinated extract by rotary evaporation and

Schlenk vacuum. The residual crude solid was used without purification.

Leu-Phe-Leu-Leu-Gly-Arg(Pbf)-Val-Leu-Ser(t-Bu)-Gly-Leu-Leu-NH_2. A sample was deprotected and analyzed. ES^+-MS 626 $[M + 2]^{2+}$.

General Procedure for Preparation of α-[4-(Carboxymethyl)-phenyl]azo Peptides. Carboxylic acid **15** (1 equiv), *N*,*N*′-dicyclohexylcarbodiimide (1 equiv), and 1-hydroxybenzotriazole (1 equiv) were stirred in anhydrous DMF (10.3 mL/mmol carboxylic acid) under argon for 10 min. Protected peptide (1 equiv) was added, and the mixture was stirred overnight for 1 day. The mixture was then deprotected, purified, and analyzed.

N^{α}-(α-[4-(Carboxymethyl)phenyl]diazenyl-isobutyryl)-temporin (17). Yellow solid. R_t 31.39 min.; λ_{max} 197 nm; ES^+-MS 551 $[M + 2 + Na]^{3+}$, 557 $[M + 2 + K]^{3+}$, 816 $[M + 2]^{2+}$, 826 $[M + 1 + Na]^{2+}$, 835 $[M + 1 + K]^{2+}$; HRMS ES^- TOF Calcd for $C_{80}H_{128}N_{19}O_{17}$: 1626.9736. Found: 1626.9747.

■ ASSOCIATED CONTENT

Supporting Information

Characterization spectra for compounds; crystallographic information files for compounds **2a**, **3a**, **4a**, **4b**, and **10a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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