Aspects of the Synthesis of an Exceptionally Preorganized Self-Immolative Spacer–Phenolate Unit

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Abstract: This study introduces a highly preorganized self-immolative spacer that is coupled to a nonfluorescent leaving group. The water-soluble compound can rapidly liberate a water-insoluble fluorescent precipitate by intramolecular attack of a free amine on a phenolic ester group via a six-membered transition state. One of the crucial steps in the synthesis of this molecule was a sterically very unfavored nucleophilic substitution to create a tertiary ether; this was optimized using model compounds. Another key challenge was deprotection by catalytic hydrogenation at low temperatures in order to furnish the hydroacetate of the free amine without further fragmentation. LC-MS and fluorescence studies showed that this compound collapsed instantaneously in methanol, as well as within a wide pH range in buffered aqueous media.

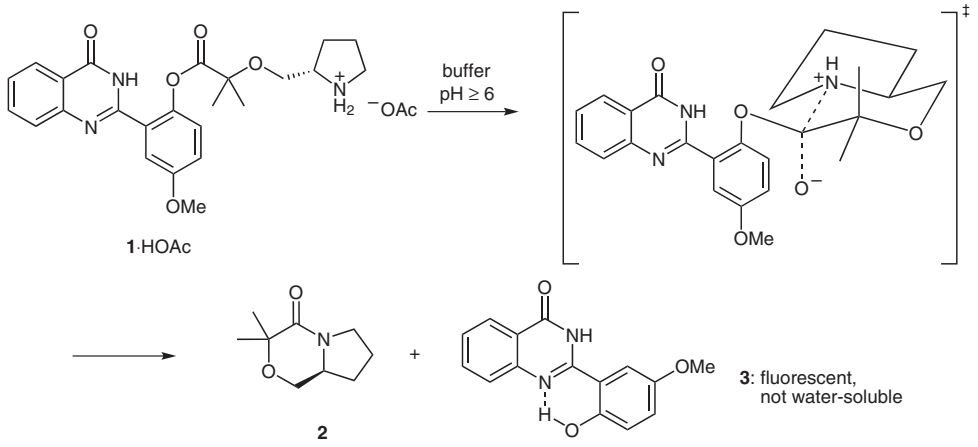
Key words: self-immolative spacer, preorganization, tertiary ether formation, low temperature hydrogenation, fluorescence

The concept of self-immolative spacers has found wide application in the domain of prodrug activation[2] and in the development of enzyme assays.[3] Agar plate based assays have proved to be suitable for the high-throughput screening of large candidate libraries. Probes that yield fluorescent precipitates (rather than colorimetric ones) directly on the agar plate may allow highly sensitive tests to be established. Such a format becomes possible if the probe molecule is water-soluble while the liberated fluorophore is not, thereby effectively repressing diffusion and thus signal dilution.

In our ongoing research on the design of fluorogenic probes, we were interested in developing an efficient synthesis of molecule **1** that, it was hoped, would rapidly fragment in aqueous media due to its highly preorganized structure, thus liberating lactam **2** and fluorescent precipitate **3** (Scheme 1).

Compound **3**, the methoxy derivative of 2-(2-hydroxyphenyl)quinazolin-4(3*H*)-one (HPQ), is rare in its properties; it is both water-insoluble and highly fluorescent in the solid state.[4] Both its fluorescence and insolubility are intimately tied to internal hydrogen bonding between the phenolic hydrogen and the imine nitrogen.[5] However, by masking the phenol group, compounds can be obtained that are water-soluble and not fluorescent.

Central to this project is the strong preorganization of the spacer unit to guarantee fast, in an ideal case instantaneous, fragmentation. One of the two preorganizational elements is the rotationally restricted N–C bond being part of a pyrrolidine moiety that is derived from proline. The second and synthetically more challenging element to raise preorganization is the quaternary center α to the phenolic ester, which constitutes an exploitation of the Thorpe–Ingold effect. This geminal dimethyl motive was also chosen to suppress ester hydrolysis, which is known to be catalyzed intramolecularly by the imine nitrogen of HPQ.[6]



Scheme 1 Fragmentation of fluorophore-spacer unit **1** in aqueous media, liberating fluorescent precipitate **3**

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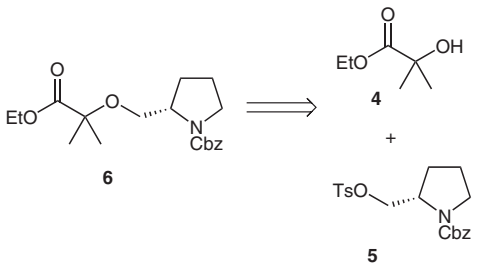
Establishing the quaternary carbon was one of the key challenges in the synthesis of **1**. Although there might be different strategies for its formation, such as ester enolate alkylation,[7] we envisaged a nucleophilic substitution reaction as shown in Scheme 2.



Scheme 2 Retrosynthetic assembly of tertiary ether **6**

While it is obvious that this transformation is sterically demanding, it has the advantage of furnishing the already relatively complex ether **6** in only one step from commercial **4** and readily available **5**. The literature only mentions sterically very hindered ether-forming reactions when phenolates are used as nucleophiles, for example, in the synthesis of clofibrate analogues[8] or in the formation of benzoxazines.[9] However, if the alcohol is aliphatic, and hence less nucleophilic, literature examples are rare. Probably the classic knowledge that such reactions are sterically disfavored has discouraged their inclusion in synthetic schemes and attempts for optimization have not been made. In fact, we found only one study where such a tertiary ether unit is formed in low yield by reaction of a rather inert alcohol with a tertiary bromide.[10] Our approach represents the opposite sense of attack of a tertiary alcohol on a primary tosylate.

The second challenge in this study was the deprotection and isolation of compound **1** in order to test its cyclization tendency in aqueous buffered media. In this particular case, removal of the benzyloxycarbonyl (Cbz) group by catalytic hydrogenation was not trivial, regarding the high (in the other context of this project very much desired) tendency of the free amine to intramolecularly attack the activated phenol ester. Conditions had to be found that would allow deprotection, yet would prevent cyclization.

This study describes the synthesis of **1**, the optimization of two interesting key steps, and finally the fragmentation tendency of the spacer unit at different pH values in buffered aqueous media as followed by LC-MS and fluorescence spectroscopy.

Initially we found that the phenolic ester of model compound **7** is easily hydrolyzed, which is in accord with a previous report (Figure 1).[6] These authors proposed that this effect is due to anchimeric assistance of the neighboring imine moiety in HPQ. When we used pivaloylated HPQ **8** instead, no background hydrolysis could be observed. Apparently, the quaternary center α to the phenolic ester carbonyl prevents access by the imine nitrogen to the ester carbonyl and, thus, its assistance in hydrolysis.

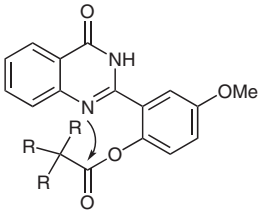
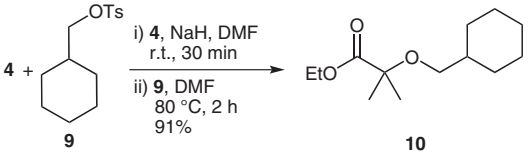


Figure 1

Subsequently, compound **5** (Scheme 2) was synthesized by Cbz protection,[11] followed by tosylation[12] of commercially available (*S*)-prolinol. Treatment of **4** with sodium hydride, followed by reaction with **5** gave, not unexpectedly, complex product mixtures that were difficult to analyze. NMR and LC-MS analysis of these mixtures led us to conclude that the carbamate function was involved in side reactions. Therefore, we decided to use **9** as a model compound, which should make it easier to analyze and optimize this reaction (Scheme 3).

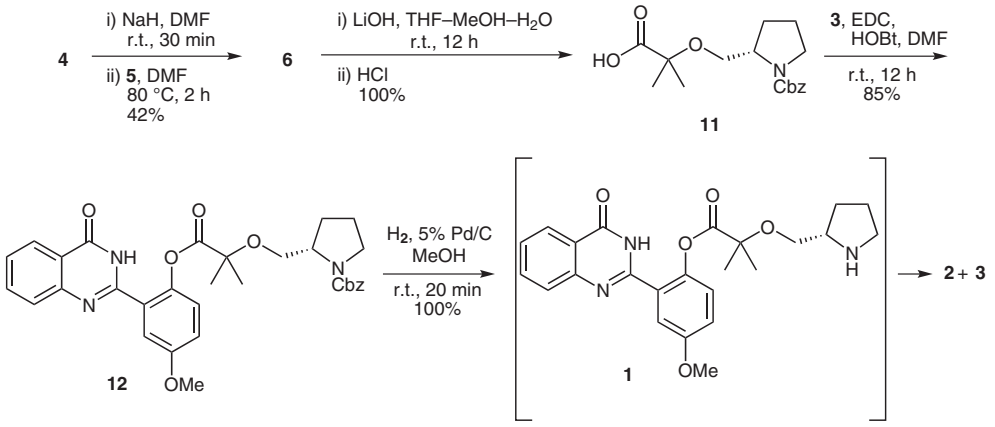


Scheme 3 Model reaction, using **9** to form tertiary ether **10**

Compound **4** was thus deprotonated with sodium hydride and reacted under various conditions with **9**, that, in return, was obtained by tosylation[12a] of commercial cyclohexylmethanol. The results of this investigation can be summarized as follows: In tetrahydrofuran, no reaction took place at all, either by refluxing the mixture, or by extending the reaction time, or by the use of an excess of sodium hydride. In *N*,*N*-dimethylformamide, no conversion was observed at room temperature. However, by raising the temperature to about 80 °C the transformation became surprisingly fast, yielding **10** in only two hours in high yield. To our knowledge, formation of an aliphatic ether at a quaternary center in high yield has not yet been described. Our results demonstrate that in spite of its steric demand this reaction can be effectively conducted at reasonable temperatures, provided that additional functional groups present in the molecule are compatible.

The conditions worked out in the preparation of **10** were then applied to the reaction of **4** with **5** (Scheme 4). As expected, the yield of **6** was lower than the one for **10**, likely because of involvement of the carbamate function as already mentioned. Still, using the optimized reaction conditions from above, a rather satisfactory yield of 42% is obtained. For comparison, a yield of only 15% was achieved in the only literature example of comparable steric demand.[10]

In the following, compound **6** was hydrolyzed[13] to give quantitatively the corresponding acid **11**. Reaction with



Scheme 4 Synthesis of **12** and in situ fragmentation via **1** after deprotection in methanol

3[4,5] using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide and 1-hydroxy-1*H*-benzotriazole in *N*,*N*-dimethylformamide as coupling agents[14] furnished **12** in 85% yield. Catalytic hydrogenation of **12** using 5% palladium-on-carbon at room temperature in methanol directly produced compounds **2** and **3** in only 20 minutes. The free amine intermediate **1** could not be trapped under these conditions, demonstrating the high cyclization tendency of the spacer unit, and thus corroborating our molecular design.

We then evaluated the performance of **1** in buffered aqueous media. Rapid fragmentation was by no means guaranteed considering the pK_a of prolinol (10.09),[15] corresponding to only 0.08% of compound **1** in its unprotonated form at pH 7. We therefore intended to isolate **1**, in order to test its behavior at different pH values in buffered aqueous media. To achieve this, deprotection of **12** had to be carried out under conditions that would prevent the highly favored cyclization. We speculated that deprotection under acidic conditions would considerably decrease the nucleophilicity of the pyrrolidinyl nitrogen and thus inhibit fragmentation. Another possible strategy was to conduct the hydrogenation at low temperatures. The results of our extensive investigation into this deprotection process, using LC-MS for reaction monitoring, are shown in Table 1, and can be summarized as follows: (1) In contrast to the use of 5% palladium-on-carbon, the use of 10% palladium-on-carbon at room temperature led to partial reduction of the imine function in the heterocycle. However, at low temperatures this side reaction was not observed, even with an increased amount of 10% palladium-on-carbon. (2) The use of acidic conditions did not prevent partial fragmentation at room temperature or at 0 °C. When deprotection was complete after 20 minutes, a small amount of **2** was already formed. (3) Analysis at meticulously adjusted temperatures proved that the deprotection was sufficiently fast at –20 °C. Below –30 °C, no reaction took place at all, not even with an increased amount of catalyst. (4) deprotection at –20 °C with 10 mol% of 5% palladium-on-carbon was incomplete after six hours. During this time, however, a considerable proportion of **1** underwent cyclization to give **2**. Increasing the amount of catalyst fourfold accelerated the reaction significantly. However, when deprotection was complete

Table 1 Optimization of the Reaction Conditions for the Conversion of **12** into **1**·HOAc

Entry	Solvent	Catalyst	Temp (°C)	Time (min)	Products[a]
1	MeOH	10% Pd/C, (10 mol%)[b]	r.t.	20	**2**[c]
2	MeOH	5% Pd/C, (10 mol%)	r.t.	20	**2**
3	MeOH–H_2O–AcOH	5% Pd/C, (10 mol%)	r.t.	20	**1**, **2**
4	MeOH–H_2O–AcOH	5% Pd/C, (10 mol%)	0	20	**1**, **2**
5	MeOH–H_2O–AcOH	5% Pd/C, (10 mol%)	< –30	360	–
6	MeOH–H_2O–AcOH	5% Pd/C, (40 mol%)	< –30	360	–
7	MeOH–H_2O–AcOH	5% Pd/C, (10 mol%)	–20	360	**1**, **12**, **2**
8	MeOH–H_2O–AcOH	5% Pd/C, (40 mol%)	–20	180	**1**, **2**
9	MeOH–H_2O–AcOH	10% Pd/C, (80 mol%)	–20	90	**1**

[a] Compounds **2** and **3** are formed simultaneously, but to simplify the table only **2** is listed.
[b] mol% is the mole percentage of Pd in relation to **12**.
[c] Partial imine reduction in the heterocycle was observed.

after three hours a small quantity of **2** could still be detected. Finally, by doubling the palladium content (i.e., 10% Pd/C), deprotection was complete in only 90 minutes without any detectable trace of **2**. Careful evaporation of the solvent at low temperature furnished the pure hydroacetate of **1**. This investigation presents a precise correlation between temperature, amount of catalyst, and reaction time. It may be helpful for other cases where Cbz deprotection is accompanied by side reactions under standard conditions.

In order to test the cyclization tendency of **1** in aqueous media, it was dissolved in phosphate buffer at pH 4, where it was stable. Subsequently, the pH was increased by adding aqueous sodium hydroxide and a sample was taken from this solution and immediately analyzed by LC-MS (Figure 2). This way we proved that **1** underwent instantaneous cyclization at pH 7 and 8, thus showing the same behavior as in nonacidified methanol (Scheme 4).

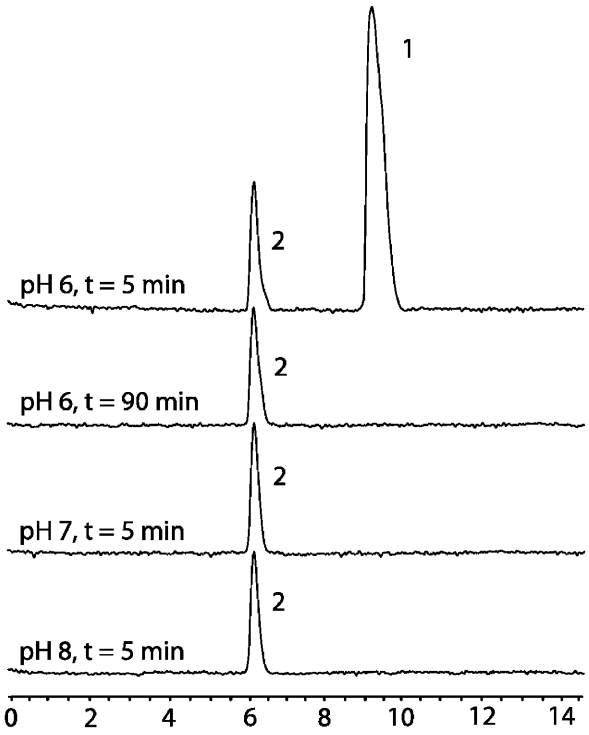


Figure 2 LC-MS monitoring of the fragmentation of **1** at different pH values; precipitate **3** is filtered off prior to injection into the LC-MS apparatus, and does therefore not appear in the chromatogram

Even at pH 6, 25% of **1** was already cyclized after 5 minutes and analysis after 90 minutes showed formation of **2** to be complete. These results are particularly striking in view of the traces of the pyrrolidine nitrogen actually existing in its unprotonated form at this pH and, thus, illustrate the pronounced preorganization of the spacer.

The fragmentation process was also monitored by fluorescence spectroscopy. The water-insoluble HPQ derivative **3** is known to emit a strong fluorescence signal at $\lambda = 550$ nm with an excitation maximum at $\lambda = 370$ nm.[5] After increasing the pH of the initial buffer solution (see above) to the desired value, a sample was immediately subjected to fluorescence analysis. Figure 3 shows the three curves that were obtained at different pH values. At pH 6, a sigmoidal curve was observed that reached a plateau after 30 minutes. At pH 7 and 8 the initial value did not show any evolution over time which can be explained with the immediate and complete liberation of fluorophore **3**. In all three cases, the release of the water-insoluble fluorophore was characterized by the solution turning cloudy. This behavior at physiological pH is in accord with the results from the LC-MS analyses.

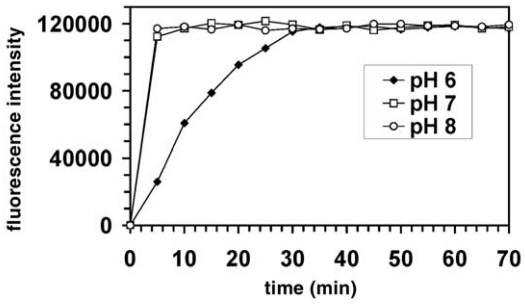


Figure 3 Fluorescence monitoring of the fragmentation of **1** releasing **3** at different pH values

In conclusion, we have conceived a highly preorganized self-immolative spacer that fragments spontaneously in methanol and in aqueous media to liberate a fluorescent precipitate based on the distinctive HPQ system. Application of this spacer is, of course, not limited to the generation of free HPQ, but may well be extended to the liberation of other leaving groups attached to the carbonyl. Its synthesis required us to optimize a sterically very unfavored case of ether formation. The elevated yields obtained in this study are so far unique in the literature and the observations made during reaction optimization may turn out to be of use in very different synthetic contexts. The same holds true for our results in conducting a catalytic hydrogenation at low temperature in order to prevent unwanted side reactions of our target compound. Finally, our observation of the instantaneous fragmentation of the spacer unit at a wide pH range provide precious insight into the possibilities in designing new probes for detection and imaging.

Reagents and solvents were purchased from Aldrich, Acros, and Alfa Aesar. HPQ derivative **3**[5] and Cbz-(*S*)-prolinol[11] were prepared as previously described. THF was distilled from Na/benzophenone, and pyridine was dried using KOH prior to distillation. Column chromatography was performed using Merck silica gel Si 60 (40–63 μm). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity 500 spectrometer (499.83 and 126.7 MHz, respectively), or a Bruker DPX 200 instrument (200.13 and 50.13 MHz, respectively). Chemical shifts are referenced from TMS or from solvent references.[16] HRMS were recorded by the Service Central d'Analyse du CNRS, Solaize, France. Melting point determinations were carried out on a Büchi melting point B-540 apparatus and are uncorrected. For those compounds existing as a mixture of rotamers, the signal

corresponding to equivalent protons or carbons of different rotamers is separated by 'and'.

4-Methoxy-2-(4-oxo-3,4-dihydroquinazolin-2-yl)phenyl Acetate (7)

Compound **3** (120 mg, 0.45 mmol) was dissolved in anhyd pyridine (5 mL) and Ac_2O (420 μL, 4.5 mmol) was added. The reaction was refluxed for 2 h then brought to r.t. and quenched by the addition of H_2O (5 mL). The mixture was extracted with Et_2O (3 × 5 mL) and the combined organic extracts were dried (Na_2SO_4) and the solvent was removed under vacuum. Compound **7** was obtained as a white solid after recrystallization (cyclohexane–EtOAc); yield: 119 mg (85%); mp 194 °C.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 10.40 (br s, 1 H), 8.27 (d, *J* = 7.7 Hz, 1 H), 8.00 (d, *J* = 7.7 Hz, 1 H), 7.78–7.72 (m, 2 H), 7.54–7.39 (m, 2 H), 7.24 (d, *J* = 7.5 Hz, 1 H), 3.82 (s, 3 H), 2.28 (s, 3 H).

^{13}C NMR (50 MHz, $CDCl_3$): δ = 168.8, 162.3, 149.7, 149.1, 148.4, 134.9, 132.3, 130.5, 128.0, 127.2, 126.7, 126.5, 126.0, 123.8, 121.0, 57.5, 21.1.

HRMS (ESI): *m*/*z* [M + H]$^+$ calcd for $C_{17}H_{15}N_2O_4$: 311.1032; found: 311.1035.

4-Methoxy-2-(4-oxo-3,4-dihydroquinazolin-2-yl)phenyl 2,2-Dimethylpropanoate (8)

Following the typical procedure for **7** using **3** (120 mg, 0.45 mmol) with pivalic anhydride (770 μL, 4.5 mmol) in pyridine (5 mL) gave **8** as a white solid after recrystallization (cyclohexane–EtOAc); yield: 141 mg (89%); mp 162 °C.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.71 (br s, 1 H), 8.30 (d, *J* = 7.7 Hz, 1 H), 8.03 (d, *J* = 7.7 Hz, 1 H), 7.82–7.76 (m, 2 H), 7.53–7.41 (m, 2 H), 7.17 (d, *J* = 7.5 Hz, 1 H), 3.84 (s, 3 H), 1.34 (s, 9 H).

^{13}C NMR (50 MHz, $CDCl_3$): δ = 176.4, 162.5, 150.1, 149.0, 148.7, 134.7, 131.9, 130.4, 127.8, 126.9, 126.8, 126.4, 126.2, 123.3, 120.8, 57.7, 39.1, 26.9.

HRMS (ESI): *m*/*z* [M + H]$^+$ calcd for $C_{20}H_{21}N_2O_4$: 353.1502; found: 353.1504.

Benzyl (*S*)-2-(Tosyloxymethyl)pyrrolidine-1-carboxylate (5); Typical Procedure

Cbz-(*S*)-prolinol[11] (11.20 g, 47.66 mmol) was dissolved in anhyd pyridine (80 mL), and TsCl (27.00 g, 142.11 mmol) was added at 0 °C. The temperature was allowed to rise up to r.t. overnight and was quenched with H_2O (20 mL). EtOAc (80 mL) was added and the mixture was washed with 1 M HCl (2 × 20 mL), H_2O (20 mL), and brine (20 mL). The organic phase was dried (Na_2SO_4), and the solvent was removed under vacuum. After flash chromatography (cyclohexane–EtOAc, 1:1), pure **5** was obtained as a white solid; yield: 17.06 g (92%); mp 51 °C.

$[\alpha]_D^{25}$ –42.6 (*c* 1.02, $CHCl_3$).

^{1}H NMR spectral data were in accord with the literature values,[12b] but HRMS and ^{13}C NMR data were not reported previously.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.79–7.69 (m, 4 H), 7.37–7.24 (m, 14 H), 5.10–4.97 (m, 4 H), 4.20–3.97 (m, 6 H), 3.42–3.32 (m, 4 H), 2.42 (s, 6 H), 2.00–1.81 (m, 8 H).

^{13}C NMR (50 MHz, $CDCl_3$): δ = 154.6 and 154.3, 144.7, 136.5 and 136.3, 132.73 and 132.67, 129.7, 128.4, 127.9, 127.7, 127.6, 69.7 and 69.6, 66.8 and 66.6, 56.0 and 55.3, 46.9 and 46.6, 28.4 and 27.5, 23.7 and 22.7, 21.5.

HRMS (ESI): *m*/*z* [M + H]$^+$ calcd for $C_{20}H_{24}NO_5S$: 390.1376; found: 390.1378.

Cyclohexylmethyl Tosylate (9)

Following the typical procedure for **5** using commercial cyclohexylmethanol (2.00 g, 17.5 mmol) and TsCl (10.00 g, 52.6 mmol) in anhyd pyridine (30 mL) gave **9** as a white solid after recrystallization (cyclohexane); yield: 3.55 g (76%); mp 32 °C (Lit.[12a] 30–32 °C).

^{13}C NMR spectral data were in accord with literature values,[12a] but HRMS and ^{1}H NMR data were not reported previously.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.78 (d, *J* = 8.2 Hz, 2 H), 7.34 (d, *J* = 8.2 Hz, 2 H), 3.81 (d, *J* = 6.4 Hz, 2 H), 2.45 (s, 3 H), 1.66–1.59 (m, 6 H), 1.26–1.18 (m, 3 H), 0.93–0.86 (m, 2 H).

^{13}C NMR (50 MHz, $CDCl_3$): δ = 144.6, 133.4, 129.7, 127.9, 75.3, 37.5, 29.4, 26.2, 25.4, 21.7.

HRMS (ESI): *m*/*z* [M + H]$^+$ calcd for $C_{14}H_{21}O_3S$: 269.1212; found: 269.1213.

Ethyl 2-(Cyclohexylmethoxy)-2-methylpropanoate (10); Typical Procedure

NaH (169 mg, 60% in mineral oil, 4.23 mmol) was suspended in DMF (20 mL) and the mixture was cooled to 0 °C. Commercial alcohol **4** (500 μL, 3.67 mmol) was added, and the mixture was brought up to r.t. and stirred for 30 min. Compound **9** (984 mg, 3.67 mmol) in DMF (5 mL) was added, and the mixture was heated to 80 °C for 2 h. The mixture was cooled to 0 °C, quenched with sat. aq NH_4Cl (20 mL), and extracted with Et_2O (3 × 25 mL). The combined organic extracts were washed with brine (2 × 25 mL) and dried (Na_2SO_4) and the solvent was removed under vacuum. After purification by flash chromatography (cyclohexane–EtOAc, 9:1), **10** was obtained as a yellow oil; yield: 761 mg (91%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 4.16 (q, *J* = 7.1 Hz, 2 H), 3.13 (d, *J* = 6.4 Hz, 2 H), 1.78–1.67 (m, 6 H), 1.34 (s, 6 H), 1.28 (t, *J* = 7.1 Hz, 3 H), 1.25–1.16 (m, 3 H), 0.92–0.85 (m, 2 H).

^{13}C NMR (50 MHz, $CDCl_3$): δ = 174.8, 77.1, 70.3, 60.7, 38.4, 30.1, 26.6, 25.8, 24.6, 14.2.

HRMS (ESI): *m*/*z* [M + H]$^+$ calcd for $C_{13}H_{25}O_3$: 229.1805; found: 229.1808.

Benzyl (*S*)-2-{[1-(Ethoxycarbonyl)-1-methylethoxy]methyl}pyrrolidine-1-carboxylate (6)

Following the typical procedure for **10**, alcohol **4** (1.03 mL, 7.57 mmol) was deprotonated with NaH (348 mg, 60% in mineral oil, 8.70 mmol) in DMF (40 mL). Reaction with **5** (2.94 g, 7.57 mmol) in DMF (10 mL) gave **6** as a yellow oil after purification by flash chromatography (cyclohexane–EtOAc, 7:3); yield: 1.11 g (42%).

$[\alpha]_D^{25}$ –44.3 (*c* 1.06, $CHCl_3$).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.36–7.21 (m, 10 H), 5.18–5.04 (m, 4 H), 4.17–4.07 (m, 4 H), 4.00–3.91 (m, 2 H), 3.59 (dd, *J* = 8.2, 3.0 Hz, 1 H), 3.50–3.32 (m, 6 H), 3.24–3.20 (m, 1 H), 2.14–2.10 (m, 2 H), 2.00–1.81 (m, 6 H), 1.41 (s, 3 H), 1.37 (s, 3 H), 1.33 (s, 6 H), 1.28 (t, *J* = 7.1, 3 H) and 1.23 (t, *J* = 7.1, 3 H).

^{13}C NMR (50 MHz, $CDCl_3$): δ = 174.7 and 174.5, 154.9 and 154.3, 137.0 and 136.9, 128.4, 128.0, 127.9, 77.5 and 77.2, 66.7 and 66.5, 65.2 and 64.6, 60.8, 57.6 and 56.9, 47.0 and 46.7, 28.5 and 27.7, 24.9, 24.3, 23.7 and 22.8, 14.2.

HRMS (ESI): *m*/*z* [M + H]$^+$ calcd for $C_{19}H_{28}NO_5$: 350.1969; found: 350.1974.

Benzyl (*S*)-2-[(1-Carboxy-1-methylethoxy)methyl]pyrrolidine-1-carboxylate (11)

Ester **6** (1.15 g, 3.30 mmol) was dissolved in THF–MeOH (30 mL–40 mL), and aq 1 M LiOH (16 mL) was added. After stirring overnight at r.t., the pH value was adjusted to 2 by the addition of 1 M HCl, and the mixture was extracted with Et_2O (3 × 30 mL). The combined organic extracts were dried (Na_2SO_4) and the solvent was

removed under vacuum to give pure **11** as a white solid; yield: 1.06 g (quant.); mp 158 °C.

$[\alpha]_D^{25}$ –44.5 (*c* 1.04, acetone).

^{1}H NMR (500 MHz, acetone-d_6): δ = 7.40–7.29 (m, 10 H), 5.19–5.10 (m, 4 H), 3.98–3.90 (m, 2 H), 3.57 (dd, *J* = 8.6, 3.8 Hz, 2 H), 3.47–3.32 (m, 6 H), 1.96–1.77 (m, 8 H), 1.37 (s, 6 H), 1.33 (s, 6 H).

^{13}C NMR (50 MHz, acetone-d_6): δ = 175.8, 155.1 and 154.5, 138.3, 129.1, 128.5, 127.3, 77.8, 66.8, 65.6 and 65.2, 58.3 and 57.7, 47.3 and 47.0, 28.2 and 27.4, 24.6, 24.0, 22.9 and 22.4.

HRMS (ESI): *m/z* $[M + H]^+$ calcd for $C_{17}H_{24}NO_5$: 322.1655; found: 322.1659.

Benzyl (*S*)-2-[(1-{[4-Methoxy-2-(4-oxo-3,4-dihydroquinazolin-2-yl)phenoxy]carbonyl}-1-methylethoxy)methyl]pyrrolidine-1-carboxylate (12)

Compound **11** (500 mg, 1.56 mmol) was dissolved in DMF (20 mL), and EDC (419 mg, 2.18 mmol), then 1 M HOBt in NMM (2.34 mL, 2.34 mmol) were added. After 5 min, **3** (418 mg, 1.56 mmol) in DMF (10 mL) was added and the mixture was stirred at r.t. overnight. The reaction was quenched with sat. aq $NaHCO_3$ (20 mL) and extracted with Et_2O (3 × 20 mL). The combined organic extracts were washed with 1 M HCl (20 mL) and brine (2 × 20 mL) and dried (Na_2SO_4), and the solvent was evaporated under vacuum. Purification by flash chromatography (cyclohexane–EtOAc, 1:1) gave **12** as a colorless oil; yield: 757 mg (85%).

$[\alpha]_D^{25}$ –45.6 (*c* 1.03, acetone).

^{1}H NMR (500 MHz, acetone-d_6): δ = 11.17 (br s, 1 H), 8.20 (d, *J* = 7.8 Hz, 2 H), 7.89 (d, *J* = 7.8 Hz, 2 H), 7.76 (d, *J* = 7.6 Hz, 2 H), 7.69–7.57 (m, 4 H), 7.53–7.47 (m, 2 H), 7.40–7.26 (m, 12 H), 5.12–5.01 (m, 4 H), 4.01 (s, 6 H), 3.90–3.81 (m, 2 H), 3.60–3.55 (m, 1 H), 3.49–3.44 (m, 1 H), 3.32–3.18 (m, 6 H), 1.97–1.91 (m, 2 H), 1.81–1.66 (m, 6 H), 1.45 (s, 6 H), 1.42 (s, 6 H).

^{13}C NMR (50 MHz, acetone-d_6): δ = 173.0 and 172.8, 162.4, 155.1 and 154.9, 151.7, 149.8, 149.4, 138.3, 135.2, 132.5, 131.2, 129.1, 128.6, 128.5, 127.7 127.6, 127.0, 126.8, 123.9, 123.7, 122.2, 78.2, 66.7, 65.8 and 65.3, 58.1 and 57.5, 56.3, 47.5 and 47.1, 28.4 and 28.1, 25.4, 24.6, 23.1 and 22.8.

HRMS (ESI): *m/z* $[M + H]^+$ calcd for $C_{32}H_{34}N_3O_7$: 572.2398; found: 572.2398.

(*S*)-2-[(1-{[4-Methoxy-2-(4-oxo-3,4-dihydroquinazolin-2-yl)phenoxy]carbonyl}-1-methylethoxy)methyl]pyrrolidinium Acetate (1·HOAc)

Compound **12** (200 mg, 0.35 mmol) was dissolved in MeOH (10 mL containing 5 vol% AcOH), and 10% Pd/C (298 mg) was added. The mixture was cooled to –20 °C and stirred under a H_2 atmosphere (1 bar) for 1.5 h. Then the catalyst was filtered off, and the solvent was removed under vacuum at low temperature using an oil pump. The residual solid was further dried at r.t. to give the pure ammonium acetate of **1** as a white powder; yield: 174 mg (quant.); mp 178 °C.

$[\alpha]_D^{25}$ –47.4 (*c* 1.00, H_2O containing 10 vol% AcOH).

^{1}H NMR (500 MHz, D_2O containing 10 vol% AcOH-d_4): δ = 7.98 (d, *J* = 7.8 Hz, 2 H), 7.71 (d, *J* = 7.8 Hz, 2 H), 7.55–7.41 (m, 6 H), 7.35–7.29 (m, 2 H), 7.15–7.09 (m, 2 H), 3.92 (s, 3 H) and 3.89 (s, 3 H), 3.80–3.72 (m, 1 H), 3.68–3.54 (m, 4 H), 3.50–3.44 (m, 1 H), 2.98–2.74 (m, 3 H), 2.68–2.60 (m, 1 H), 1.74–1.65 (m, 2 H), 1.55–1.43 (m, 6 H), 1.23 (s, 6 H), 1.20 (s, 6 H).

^{13}C NMR (127 MHz, D_2O containing 10 vol% AcOH-d_4): δ = 174.1 and 173.9, 164.8, 164.0, 151.5, 147.3 and 147.0, 136.1 and 136.0, 133.0, 129.9, 128.2 and 128.0, 127.32 and 127.25, 126.1, 125.8, 125.2, 122.4 and 122.3, 119.7 and 119.5, 78.2 and 78.1, 63.2, 57.1 and 56.9, 56.1 and 55.7, 45.9 and 45.5, 27.1 and 26.7, 24.6, 23.6, 22.8 and 22.5.

HRMS (ESI): *m/z* $[M + H]^+$ calcd for $C_{24}H_{28}N_3O_5$: 438.2030; found: 438.2032.

3,3-Dimethyltetrahydro-1*H*-pyrrolo[2,1-*c*][1,4]oxazin-4(3*H*)-one (2)

5% Pd/C (113 mg) was added to a soln of **12** (300 mg, 0.53 mmol) in MeOH (15 mL). The mixture was stirred under a H_2 atmosphere (1 bar) at r.t. for 20 min, then the catalyst was filtered off and the filtrate was evaporated to dryness. Purification by flash chromatography (CH_2Cl_2–MeOH, 19:1) gave **2** as a colorless oil; yield: 90 mg (quant.).

$[\alpha]_D^{25}$ –47.1 (*c* 1.00, acetone).

^{1}H NMR (500 MHz, acetone-d_6): δ = 3.93 (dd, *J* = 11.2, 3.7 Hz, 1 H), 3.71–3.65 (m, 1 H), 3.47–3.31 (m, 3 H), 1.90–1.77 (m, 3 H), 1.45–1.37 (m, 1 H), 1.29 (s, 6 H).

^{13}C NMR (50 MHz, acetone-d_6): δ = 176.5, 79.4, 68.7, 57.5, 44.6, 28.0, 25.1, 24.8, 22.7.

HRMS (ESI): *m/z* $[M + H]^+$ calcd for $C_9H_{16}NO_2$: 170.1182; found: 170.1186.

LC-MS and Fluorescence Measurements

A 500 μM soln of compound **1**·HOAc in 50 mM phosphate buffer (pH 4) was prepared. Subsequently, the pH was increased to the desired value by adding a few drops of concd aq NaOH. Samples of the resulting solns were immediately analyzed by LC-MS or fluorescence spectroscopy. Fluorescence measurements were carried out using a microplate spectrofluorimeter (Spectramax Gemini XS, Molecular Devices) and black 96-well plates (Nunclone, Nunc Inc.). Fluorescence was monitored at 550 nm with an excitation wavelength set to 370 nm. Each point of the curves shown in Figure 3 is the average value of 5 independently performed experiments. LC-MS analyses were conducted on an Agilent 1100 Series LC/MSD apparatus using a Synergi Polar-RP 80A (Phenomenex) column (4 μm, 150 × 4.60 mm). Precipitated **3** was filtered off prior to injection into the apparatus. MeCN and H_2O (containing 0.01% HCO_2H) were used as mobile phase at a flow rate of 0.5 mL/min (gradient: *t* = 0 min, 0% MeCN; *t* = 10 min: 100% MeCN). Mass analysis was performed using ESI in scan mode.

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