Synthesis of ^{13}C-Labeled Pyrazinone Thrombin Inhibitors and Elucidation of Metabolic Activation Pathways

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In support of a research program aimed at discovering orally bioavailable thrombin inhibitors, ^{13}C- labeled 3-aminopyrazinone acetamide thrombin inhibitors have been prepared to aid in the structural elucidation of key metabolites during preclinical and clinical studies. A method for the ^{13}C-labeling of pyrazinones utilizing ^{13}C-glycine as a building block has been developed in a good overall yield. Two targets with labels at different positions have been prepared. NMR Studies not only led to the structure of a major metabolite, but also revealed a likely metabolic pathway, which will provide structural guidance for the design and synthesis of future thrombin inhibitors as well as other pyrazinone-containing compounds.

Introduction. – Thrombin is a trypsin-like serine protease that regulates platelet activation and plays an important role in the blood-coagulation cascade process [1]. Thrombin can convert fibrinogen to fibrin that ultimately combines with platelets and other components to form a blood clot [2]. Thrombosis, also known as excessive blood clotting, is a major factor in cardiovascular disease, which is blamed for nearly half of all deaths in the industrialized world annually [3]. Aberrant thrombosis is linked to disseminated intravascular coagulation (DIC) [4], deep vein thrombosis (DVT) [5], acute myocardial infarction (MI; heart attack) [6], unstable angina (serious chest pain preceding MI) [7], and pulmonary embolism [8]. Therefore, thrombin and other related coagulant agents such as factor Xa and prothrombinase (Ptase) have attracted enormous attention in pharmaceutical research [9]. Our focus at *Merck* has been the search for potent thrombin inhibitors with good oral bioavailability (OBA) and inhibition selectivity relative to trypsin [10–13]. Most early thrombin inhibitors were peptides or peptidomimetics with low OBA and poor pharmacokinetic (PK) profiles [9]. Recently, compound **1** has been identified as an efficacious thrombin inhibitors with excellent OBA and PK (*Figure*) [13], and it has advanced into Phase-I clinical trials as a once-daily oral anticoagulant drug candidate.

Absorption, distribution, metabolism, and excretion (ADME) studies of **1** resulted in isolation of several major metabolites, many of which came from the oxidation of the pyrazinone ring in **1**. Compound **2** and **3** were the synthetic targets with ^{13}C-label at the indicated position located on the pyrazinone ring; it was expected that they would show the same behavior as **1** during ADME studies. Metabolism and excretion of **1** varies considerably across species, but compound **4** (also known as 'metabolite number 1' or

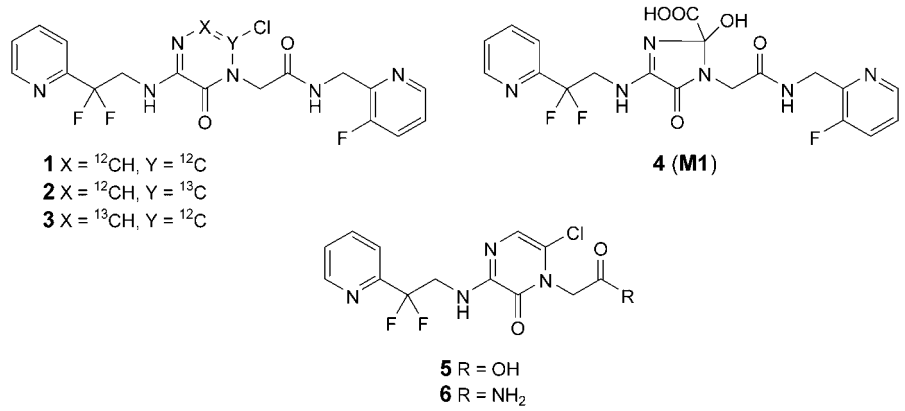
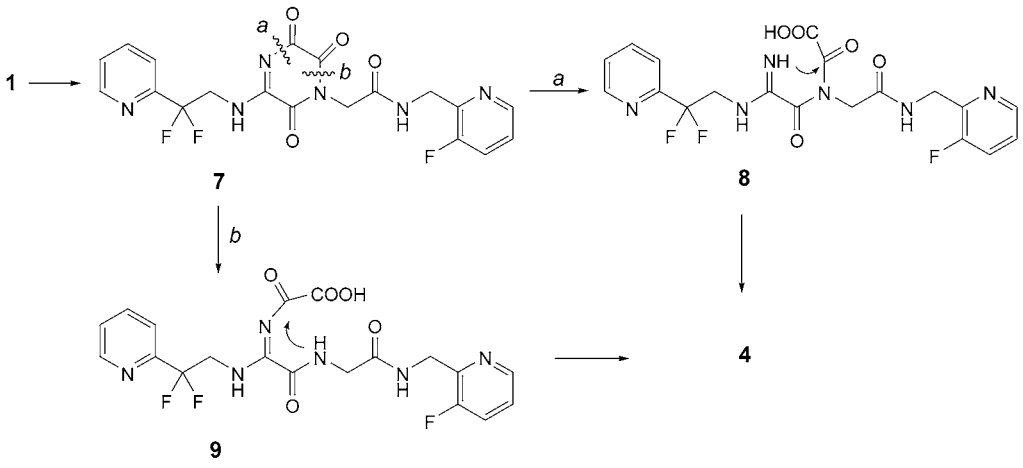


Figure. *A Non-Covalent Thrombin Inhibitor* **1** *and Its Metabolites* **4**–**6**

M1) is the predominant metabolite in all species studied (*Figure*), and its structure was determined based on both NMR and mass-spectrometric data.

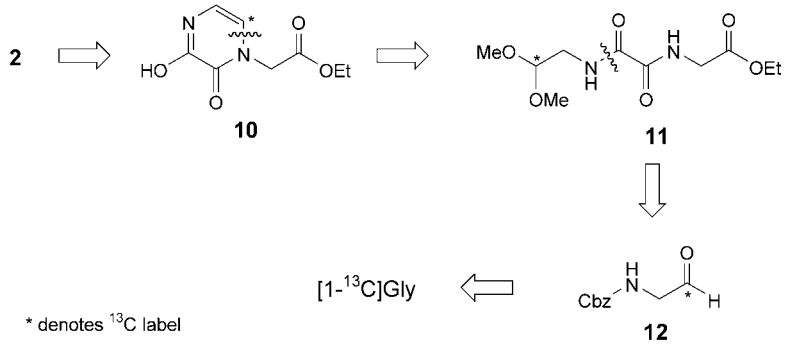
Two distinct metabolic activation pathways (*a* and *b*) were proposed (*Scheme 1*), both of which included oxidation and subsequent rearrangement of the pyrazinone heterocycle. As part of our effort to determine which pathway occurred during metabolic process, **2** and **3** were needed because metabolism of either would generate the corresponding ^{13}C-labeled metabolite, which would reveal the metabolic mechanism. These studies were important because identification of principal sites associated with drug metabolic breakdown and understanding how it happened would help us design future drug candidates containing a pyrazinone ring and to improve its duration of action, *i.e.*, PK profile.

Scheme 1. *Two Plausible Biotransformation Pathways to the Major Metabolite* **4**

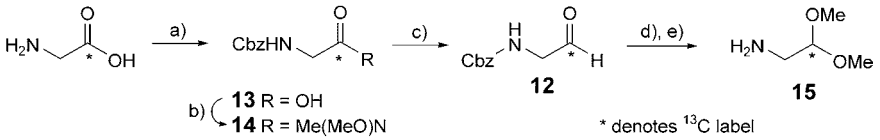


Results and Discussion. – The retrosynthetic analysis is outlined in *Scheme 2*. ^{13}C-Labeled compound **2** could be prepared from **10** (nine steps) according to the method previously developed by *Burgey et al.* [13]. The crucial pyrazinone ring would be constructed from its precursor **11**. Unlabeled compound **12** is a known one obtained by ozonolysis of the corresponding olefin. Since construction of such an olefin with the label at the desired position was not obvious, we envisioned that **12** would come from readily available [1-^{13}C]glycine. The key to this synthesis was to develop an efficient procedure to obtain the aldehyde **12**, as was shown in *Scheme 3*.

Scheme 2. *Retrosynthetic Analysis of ^{13}C-Labeled* **2**



Scheme 3. *Synthesis of Amino[1-^{13}C]acetaldehyde Dimethyl Acetal* **15**.

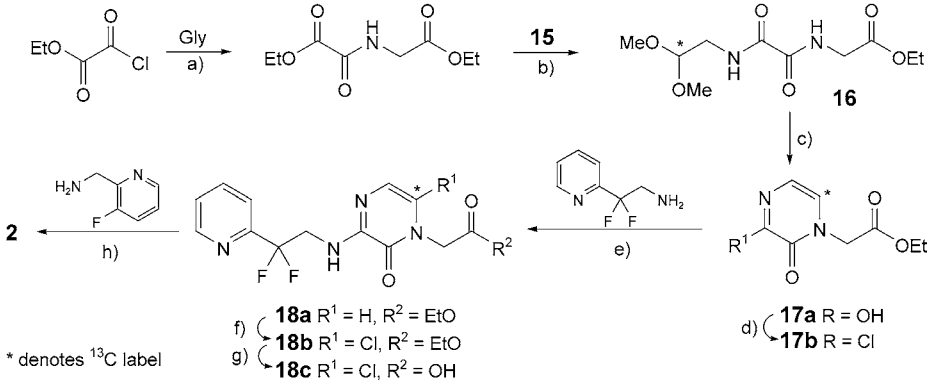


a) (Benzyloxy)carbonyl chloride (CbzCl), NaOH, H_2O, toluene, r.t., 18 h; 77%. b) *N,O*-Dimethylhydroxylamine · HCl, *N*-[3-(dimethylamino)propyl]carbodiimide · HCl (EDC), 1-hydroxy-1*H*-benzotriazole (BtOH), Et_3N, $CHCl_3$; 97%. c) $LiAlH_4$, THF, 0°; 80%. d) $HC(OMe)_3$, conc. H_2SO_4, MeOH; 95%. e) H_2, Pd/C, i-PrOH.

Acylation of [1-^{13}C]glycine with benzyl chloroformate in the presence of aqueous NaOH gave acid **13** in good yield. Reduction of **13** to the corresponding alcohol was readily accomplished but the subsequent oxidation to aldehyde **12** was found to be very difficult. Several oxidation methods were tried but they always resulted in low yields. Both the high cost of the labeled glycine and the long linear synthesis sequence (nine steps from **12** to **2**) prompted us to develop a better way to **12**. Coupling **13** with *N,O*-dimethylhydroxylamine · HCl led to the *Weinreb* amide **14** in 97% yield, and $LiAlH_4$ reduction of **14** in THF yielded aldehyde **12**. It is known that $LiAlH_4$ can also reduce the (benzyloxy)carbonyl (Cbz) protecting group but the rate of reduction is slower than that for the *Weinreb* amide. We felt that we could carry out the reduction step under kinetic control. Using an undercharged $LiAlH_4$ (90 mol-%) together with a short reaction time (0.5 h) at 0° was the best condition found for this reaction to achieve a consistently good yield (75–84%). Treatment of trimethyl orthoformate followed by

catalytic hydrogenation gave arise to 2-amino[1-^{13}C]acetaldehyde dimethyl acetal (**15**) in quantitative yield. At this point, application of the literature procedures [13] made it possible for us to obtain the final tracer **2** (*Scheme 4*). Another ^{13}C-labeled tracer **3** was prepared in the same manner with [2-^{13}C]glycine as the starting material.

Scheme 4. *Synthesis of the ^{13}C-Labeled Target* **2**

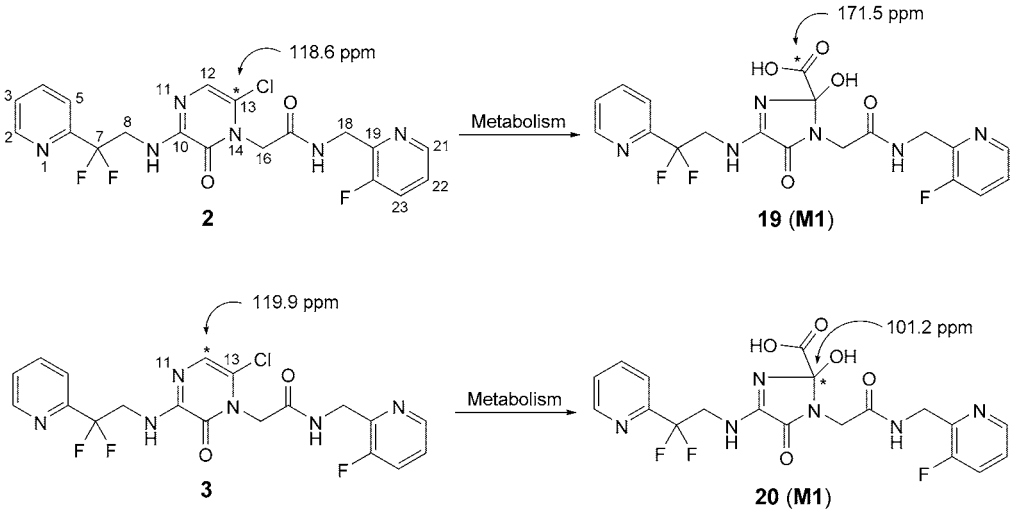


a) Glycine, Et_3N, $ClCH_2CH_2Cl$, r.t., 18 h, 95%. b) **15**, i-PrOH, r.t., 18 h, 98%. c) CF_3COOH (TFA), AcOH, reflux, 20 h. d) $(COCl)_2$, DMF, MeCOO(i-Pr), MeCN. e) $EtN(i\text{-}Pr)_2$ (DIEA), NaI, MeCN, reflux. f) NCS, MeCN. g) NaOH, THF, H_2O. h) EDC, BtOH, Et_3N.

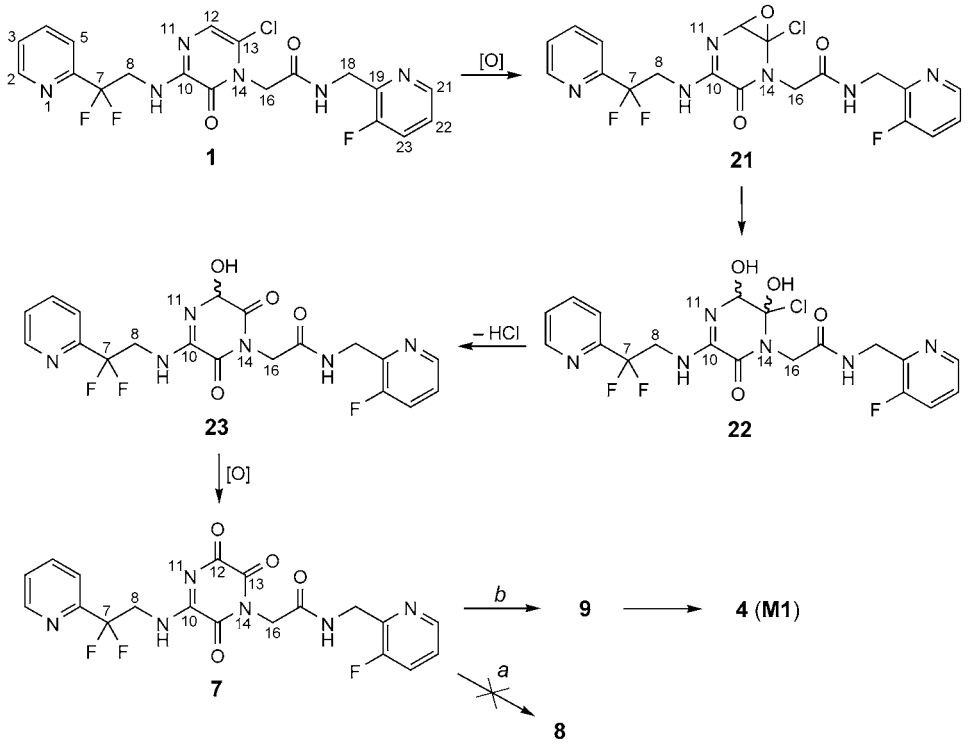
The biological assays were carried out with several important human serine proteases including the digestive enzyme trypsin, the procoagulant factor Xa, thrombin, and the thrombolytic protease plasmin. The labeled compounds **2** and **3** showed the same activity as that for unlabeled **1** [13]. For metabolic studies, **2** was administered at 20 mg/kg to bile-duct-cannulated male *Sprague–Dawley* rats. The rat bile and urine were collected at specific time intervals as required by the *in vivo* study protocol. Each metabolite was isolated with a preparative HPLC column, and the identity of the ^{13}C-labeled **M1** metabolite was confirmed by co-elution with authentic sample of **4** on an analytical column [13b].

The results are highlighted in *Scheme 5*. Compound **2** has the ^{13}C-label at position 13 whereas **3** with the label at position 12. The sole resultant **M1** metabolite from **2** turned out to be **19**, while **3** led to the isolation of **20** as the only **M1** metabolite. The label locations for **2**, **3**, **19**, and **20** were confirmed by ^{13}C-NMR spectroscopy, and this metabolic transformation was accompanied by two significant changes in the ^{13}C-NMR spectra (recorded in $CDCl_3$). In the first instance, the absorption attributable to the ^{13}C-label of **2** at position 13 (118.6 ppm) was displaced by the signal from **19** at lower field (171.5 ppm), clearly as a direct consequence of the label at the carboxylic acid C-atom. In the second case, when the metabolic experiment was performed with **3** with ^{13}C-label at position 12 (119.9 ppm), its metabolite **20** (**M1**) revealed that the label was moved to higher field (101.2 ppm), which was assigned as a OH-bearing C-atom. Based on these results and other evidence for biotransformation involving pyrazinone-ring oxidation on similar systems [14], we proposed a mechanism (*Scheme 6*) for the formation of metabolite **M1** (compound **4**).

Scheme 5. *Metabolism of Labeled Thrombin Inhibitors* **2** *and* **3**



Scheme 6. *Proposed Mechanism for the Formation of Metabolite* **M1**



This mechanism excluded the pathway *a* and was consistent with pathway *b* (*Scheme 1*), in which the pyrazinone-ring opening and contraction occurred at the C–N bond between the Cl-bearing C-atom and its neighboring N-atom in **1**. In other words, the metabolic pathway involved breaking the C–N bond between position 13 and 14, which indicates that this bond is weaker than that between position 11 and 12 (*Scheme 6*).

AM1, a semi-empirical quantum-mechanical calculation [15], was performed to understand the energetic difference between the two proposed intermediate structures **8** and **9**. Atoms from position 9 to 15 of **1** in *Scheme 6* are called the core, and they were kept free during energy minimization, except that distance constraints of 2.5 Å were applied between the atoms N(11) and C(13) in structure **8**, and N(14) and C(12) in structure **9**. The energy minimizations were carried out until the geometries converged. The results are very interesting. Not only the free energy of the non-core fragments in **8** and **9** were exactly the same, but they also were completely fixed during the energy-minimization process. Therefore, the energy difference between the minimized structures comes only from the different cores of **8** and **9**. The final energies for **8** and **9** are −220.36 and −227.46 kcal/mol, respectively. In other words, the calculation results indicated that path-*b* intermediate **9** is by *ca.* 7 kcal/mol more stable than path-*a* intermediate **8**. Since the experimental observations are clearly supportive for path *b* over *a*, the mechanism of this pyrazinone-ring-opening reaction must have involved late (product-like) transition states for the slow steps of the hydrolyses.

Conclusions. – We reported an efficient synthesis of the ^{13}C-labeled amino-acetaldehyde dimethyl acetal **15**, which was utilized to construct a labeled thrombin inhibitor for an ADME study to elucidate an important metabolic mechanism. A mechanism for the formation of metabolite **M1** (also known as **4**) from **1** was proposed based on the experimental results, which were further strengthened by AM1 molecular calculations. This study can provide some guidance on structural design for drugs candidates with a pyrazinone motif.

Experimental Part

General. All reactions were conducted under N_2. Column chromatography (CC) was performed with 230–400-mesh silica gel. Unless otherwise noted, all NMR data were obtained at 400 MHz for ^{1}H and 100 MHz for ^{13}C, in $CDCl_3$ with TMS as an internal standard.

N-*[(Benzyloxy)carbonyl][1-^{13}C]glycine* (**13**) Benzyl chloroformate (15 ml, 100 mmol) was added to an ice-cold soln. of [1-^{13}C]glycine (5.0 g, 67 mmol) in NaOH (5.0M; 50 ml, 250 mmol) and toluene (50 ml). The mixture was stirred vigorously at r.t. for 18 h. The toluene layer was separated, and the aq. soln. was cooled and acidified with conc. HCl to pH 2. The precipitate was filtered off, washed with one volume of cold H_2O, and dried. Pure **13** was obtained (13.2 g, 95%). ^{1}H-NMR ((D_6)DMSO): 3.68 (*t*, *J* = 5.6, 2 H); 5.04 (*s*, 2 H); 7.36 (*m*, 5 H); 7.57 (br. *s*, NH); 12.70 (br. *s*, OH). ^{13}C-NMR ((D_6)DMSO): 41.67; 65.50; 127.76; 127.85; 128.40; 137.06; 156.55; 171.61. MS: 211 ($[M+H]^+$), 234 ($[M+Na]^+$).

2-[[(Benzyloxy)carbonyl]amino]-N-*methoxy*-N-*methyl[1-^{13}C]acetamide* (**14**). A mixture of **13** (5 g, 23.9 mmol), EDC (4.9 g, 25.6 mmol), HOBt (3.9 g, 25.5 mmol), and Et_3N (13 ml, 9.4 g, 93.4 mmol) in $CHCl_3$ (250 ml) was stirred at r.t., while *N,O*-dimethylhydroxylamine hydrochloride (2.5 g, 25.6 mmol) was added. The resulting mixture was stirred at r.t. for 18 h under N_2. The mixture was washed with H_2O (250 ml), aq. $NaHCO_3$ (250 ml), 1M HCl (250 ml), and brine (50 ml), and dried (Na_2SO_4). Evaporation of the solvent afforded a yellow residue, which was purified by FC (AcOEt/hexanes 1:1) to give **14** (5.87 g, 97%). White solid. ^{1}H-NMR: 3.20 (*s*,

3 H); 3.71 (*s*, 3 H); 4.15 (*d*, $J = 4.0$); 5.13 (*s*, 2 H); 5.61 (br. *s*, 1 H); 7.35 (*m*, 5 H). ^{13}C-NMR ($CDCl_3$): 32.27; 40.03; 61.39; 66.79; 127.96; 128.01; 128.42; 136.36; 156.28; 169.67. MS: 254.1 ($[M+H]^+$).

2-{[(Benzyloxy)carbonyl]amino}[1-^{13}C]acetaldehyde (**12**) $LiAlH_4$ (1.0M in THF; 18 ml, 18.0 mmol) was added to a mixture of **14** (5.04 g, 20.0 mmol) in THF (150 ml) at 0° under N_2. The mixture was stirred at 0° for 1 h. A soln. of $KHSO_4$ (3.44 g, 25.3 mmol) in H_2O (80 ml) was added, and THF was removed under reduced pressure. The residue was extracted with $CHCl_3$ (4 × 75 ml). The combined org. layers were washed with 1M aq. HCl soln., $NaHCO_3$ (100 ml), and brine (100 ml), and dried ($MgSO_4$). Evaporation of the solvent in a rotary evaporator gave **12** (3.14 g, 80%). ^{1}H-NMR ($CDCl_3$): 4.04 (*d*, $J = 5.2$, 2 H); 5.11 (*s*, 2 H); 5.65 (br. *s*, 1 H); 7.30 (*m*, 5 H). ^{13}C-NMR ($CDCl_3$): 57; 62.14; 128.12; 128.24; 128.48; 136.33; 156.28; 196.46. MS: 195.1 ($[M+H]^+$).

2-Amino[1-^{13}C]acetaldehyde Dimethyl Acetal (**15**). A mixture of **12** (1.3 g, 5 mmol), trimethyl orthoformate (20 ml), MeOH (10 ml), and conc. H_2SO_4 (one drop) was stirred at r.t. for 2 d. K_2CO_3 (0.5 g) was added, and, after stirring for 1 h, the solid was filtered off, and the filtrate was used without further purification. An anal. sample was purified by FC (Et_2O/pentane 1:1). ^{1}H-NMR ($CDCl_3$): 3.28 (*dd*, $J = 139.2$, 5.4, 2 H); 3.35 (*s*, 3 H); 3.36 (*s*, 3 H); 4.36 (*t*, $J = 5.4$, 1 H), 4.61 (br. *s*, 1 H); 5.08 (*s*, 2 H); 7.35 (*m*, 5 H). ^{13}C-NMR ($CDCl_3$): 44.09; 680.7; 104.36; 129.44; 129.52; 129.98; 138.47; 157.81. MS: 255.1 ($[M+H]^+$).

Ethyl 2-({2-[(2,2-Dimethoxy[2-^{13}C]ethyl)amino]-1,2-dioxoethyl}amino)acetate (**16**). Ethyl 2-[(2-ethoxy-1,2-dioxoethyl)amino]acetate (1.1 g, 5.5 mmol) and Et_3N (0.77 ml, 5.6 mmol) were added to an i-PrOH soln. of **15** (4.8 mmol). The resulting mixture was stirred at r.t. for 18 h, concentrated, and purified by FC (hexane/AcOEt 85:15) to yield **16** (2.1 g, 98%). Oil. The spectral data are in agreement with those reported in the literature [13a]. MS: 423.1 ($[M+H]^+$).

*6-Chloro-3-{[2,2-difluoro-2-(pyridin-2-yl)ethyl]amino}-1-({*N*-[(3-fluoropyridin-2-yl)methyl]carbamoyl}-methyl) [6-^{13}C]pyrazin-2(1*H*)-one* (**2**). [^{13}C]-labeled-**16** (2 g) was converted to **2** (123 mg, 6% overall yield for six steps) using literature procedure [13a].

*6-Chloro-3-{[2,2-difluoro-2-(pyridin-2-yl)ethyl]amino}-1-({*N*-[(3-fluoropyridin-2-yl)methyl]carbamoyl}-methyl) [5-^{13}C]pyrazin-2(1*H*)-one* (**3**). 2-[^{13}C]-glycine (5 g) was converted to **3** (114 mg) using the same method as for preparation of **2**.

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