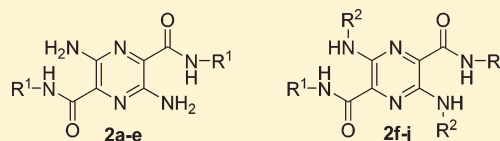


Hydrophilic Pyrazine Dyes as Exogenous Fluorescent Tracer Agents for Real-Time Point-of-Care Measurement of Glomerular Filtration Rate

Raghavan Rajagopalan,* William L. Neumann,[†] Amruta R. Poreddy, Richard M. Fitch, John N. Freskos, Bethel Asmelash, Kimberly R. Gaston, Karen P. Galen, Jeng-Jong Shieh, and Richard B. Dorshow

Covidien Pharmaceuticals, 675 McDonnell Boulevard, Hazelwood, Missouri 63042, United States

ABSTRACT: Various hydrophilic pyrazine-bis(carboxamides) derived from 3,5-diamino-pyrazine-2,5-dicarboxylic acid bearing neutral and anionic groups were prepared and evaluated for use as fluorescent glomerular filtration rate (GFR) tracer agents. Among these, the dianionic D-serine pyrazine derivatives **2d** and **2j**, and the neutral dihydroxypropyl **2h**, exhibited favorable physico-chemical and clearance properties. In vitro studies show that **2d**, **2h**, and **2j** have low plasma protein binding, a necessary condition for renal excretion. In vivo animal model results show that these three compounds exhibit a plasma clearance equivalent to iothalamate (a commonly considered gold standard GFR agent). In addition, these compounds have a higher urine recovery compared to iothalamate. Finally, the plasma clearance of **2d**, **2h**, and **2j** remained unchanged upon blockage of the tubular secretion pathway with probenecid, a necessary condition for establishment of clearance via glomerular filtration only. Hence, **2d**, **2h**, and **2j** are promising candidates for translation to the clinic as exogenous fluorescent tracer agents in real-time point-of-care monitoring of GFR.



INTRODUCTION

The assessment of renal function is a critical part of patient care, and the accurate and real-time measurement and monitoring of renal function is essential for minimizing the risk of kidney failure due to chronic or acute clinical, physiological, and pathological conditions. Glomerular filtration rate (GFR) is now widely accepted as the best indicator of renal function, and current clinical guidelines advocate its use in the staging of kidney disease.¹ Endogenous plasma creatinine assay,² and theoretical equations³ based on the plasma creatinine concentration, continue to be the most common clinical method of assessing GFR despite the known limitations.^{4–6} However, the GFR value obtained by these methods is frequently misleading because it is affected by age, state of hydration, renal perfusion, muscle mass, dietary intake, and many other anthropometric and clinical variables. Moreover, creatinine is partially cleared by tubular secretion along with glomerular filtration and, as Diskin⁵ recently remarked, “Creatinine clearance is not and has never been synonymous with GFR, and all of the regression analysis will not make it so because the serum creatinine depends upon many factors other than filtration.”

The optimum measure of GFR is by the use of exogenous tracer agents.⁷ However, the infrequently used conventional GFR agents such as inulin,⁸ iothalamate,^{9,10} and ^{99m}Tc-DTPA¹¹ suffer from various undesirable properties (such as radioactivity, need for ionizing radiation, laborious ex-vivo handling of blood and urine samples, and lack of consistent and reliable supply) that render them unsuitable for real-time point-of-care renal function monitoring. Thus, to overcome these various limitations, there

has been considerable effort directed at developing fluorescent GFR tracer agents.^{12–15}

On the basis of the considerable empirical knowledge that hydrophilic and anionic substances are preferentially cleared through the renal system,^{16,17} we had originally proposed a two-pronged approach for the rational design of fluorescent exogenous GFR tracer agents.¹³ The first method involves enhancing the fluorescence of known GFR tracer agents that are intrinsically poor fluorescent emitters such as lanthanide metal complexes, and the second involves transforming highly fluorescent dyes (which are intrinsically lipophilic) into hydrophilic, anionic species to force them to clear via the kidneys. In the first approach, we have prepared and evaluated europium–DTPA complexes endowed with various molecular “antenna” to induce ligand-to-metal fluorescence resonance energy transfer (FRET).¹³ Some success was obtained in that one of these metal complexes exhibited a 2700-fold increase in europium fluorescence (compared to Eu–DTPA) and underwent clearance exclusively through the kidneys. In this paper, we present, for the first time, the successful results of developing highly fluorescent exogenous GFR tracer agents based on the second approach.¹⁸

The key requirements for the rational design of exogenous fluorescent tracer agents are: (a) excitation and emission occurring in the visible region ($\lambda \geq \sim 425$ nm), (b) having highly hydrophilic neutral or anionic character, (c) very low or no plasma protein binding, (d) no in vivo metabolism, and (e)

Received: March 4, 2011

Published: June 13, 2011

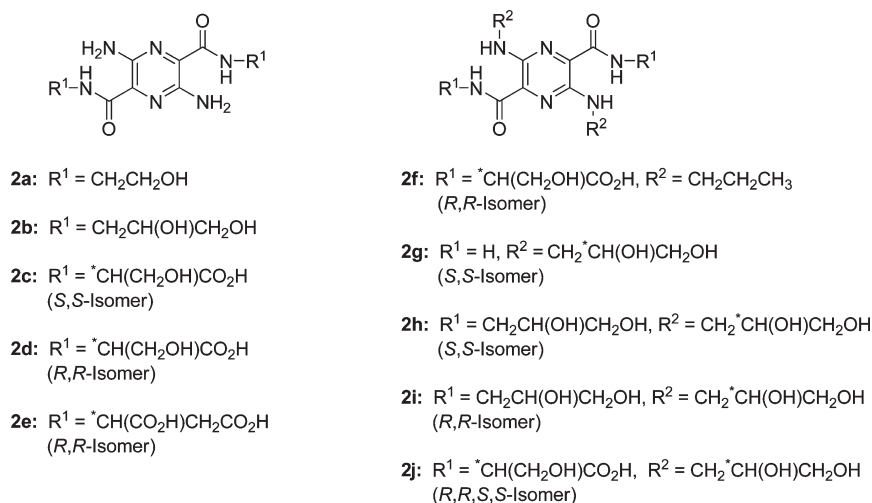
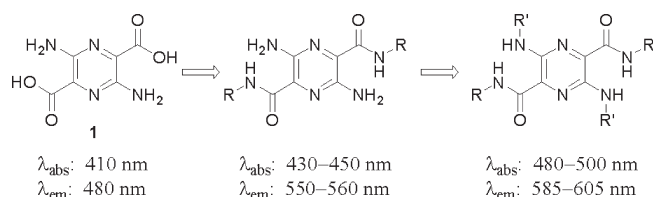


Figure 1. Pyrazine-based GFR tracer agents.

clearance exclusively via glomerular filtration (often demonstrated by equality of plasma clearance with and without a tubular secretion inhibitor such as probenecid¹⁹). The selection of the lead clinical candidate(s) may be based on secondary considerations such as the ease of synthesis, lack of toxicity, and stability. The secondary screening criteria should further take into account the tissue optics properties and the degree of extracellular distribution of the fluorescent tracers. Volume of distribution is an important parameter in the assessment of hydration state of the patient, whereas the absorption/emission properties provide essential information for the design of the probe.

Pyrazines are a class of photostable small molecules having highly desirable photophysical properties useful for in biomedical applications. Pyrazine derivatives containing electron withdrawing groups at the 2,5 positions and electron donating groups at the 3,6 positions such as 3,6-diamino-2,5-pyrazine-dicarboxylic acid (**1**) and the corresponding amides strongly absorb and emit in the blue to orange regions with a large Stokes shift on the order of ~ 100 nm and with fluorescence quantum yields of about 0.4.^{20,21} Conversion of the carboxyl group in **1** to the secondary amide derivatives produces a bathochromic shift of about 40 nm, and alkylation of the amino group results in further red-shift of about 40 nm. Hence, the pyrazine scaffold presents an attractive opportunity to “tune” the electronic and renal clearance properties at once by introducing hydrophilic substituents.



The pyrazine-dicarboxylic acid (**1**) and its known simple carboxamide analogues are lipophilic and are insoluble in water and, therefore, unsuitable as possible GFR tracer agents in this form. However, the diacid **1** is an excellent scaffold for further modification, and several hydrophilic amides bearing various neutral and anionic substituents (Figure 1) were designed,

Table 1. Absorption, Emission, and Plasma Protein Binding

compd	λ_{Abs} (nm)	λ_{Em} (nm)	PPB (%) ^a
2a	436	558	12
2b	432	558	2
2c	435	559	0
2d	435	557	0
2e	436	558	0
2f	490	599	74
2g	486	600	15
2h	484	594	6
2i	486	597	4
2j	488	597	0
iothalamate	NA ^b	NA	10 ^c

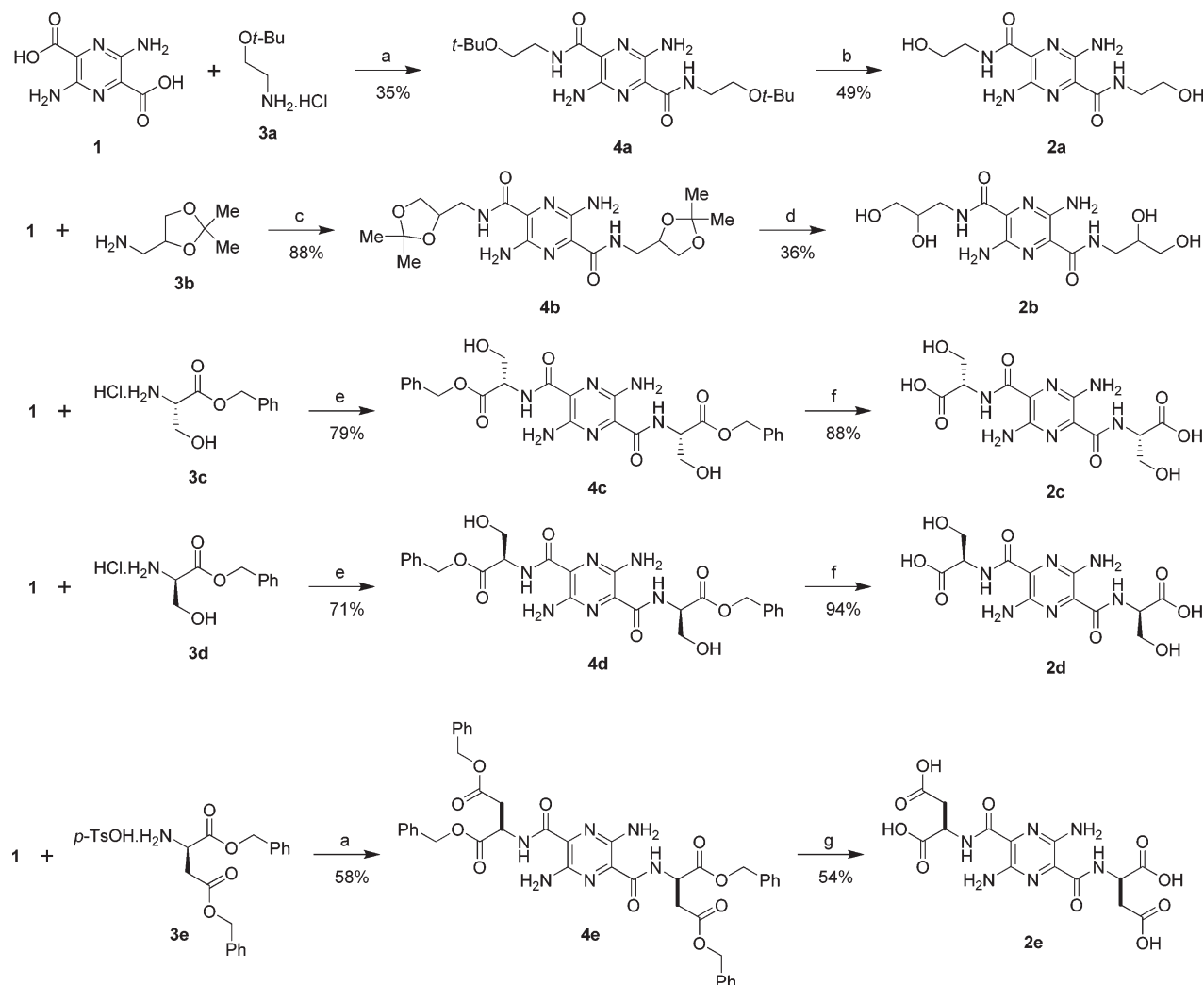
^a Measured to $\pm 1\%$. ^b Not applicable. ^c Reference 23.

synthesized, and evaluated for use as GFR tracer agent candidates. These pyrazine derivatives may be divided into two general categories. Compounds **2a–e** bear primary amino groups and absorb radiation in the blue region of the electromagnetic spectrum (Table 1). Compounds **2f–j** are N-alkylated pyrazines and absorb radiation in the green region of the spectrum.

RESULTS AND DISCUSSION

Syntheses. The neutral polyhydroxy derivatives **2a** and **2b** were prepared by condensing the diacid **1** with protected aminoethanol **3a** or aminopropanediol **3b** respectively by the standard carbodiimide coupling method, followed by deprotection with hydrochloric acid (Scheme 1). The dianionic serine derivatives **2c** and **2d** were prepared by coupling the diacid **1** with L- or D-serine benzyl ester hydrochloride (**3c** or **3d**), followed by hydrogenolysis in the presence of 10% Pd–C as catalyst at atmospheric pressure. The tetraanionic aspartate derivative **2e** was prepared by coupling the diacid **1** with dibenzyl aspartate **3e**, followed by transfer hydrogenation with ammonium formate and 10% Pd–C.

Alkyl substitution on the amino groups of the pyrazine scaffold **1** was known to significantly increase the wavelength of both absorption and emission maxima, but the existing base-induced alkylation methods resulted in rather poor yields of the desired

Scheme 1^a

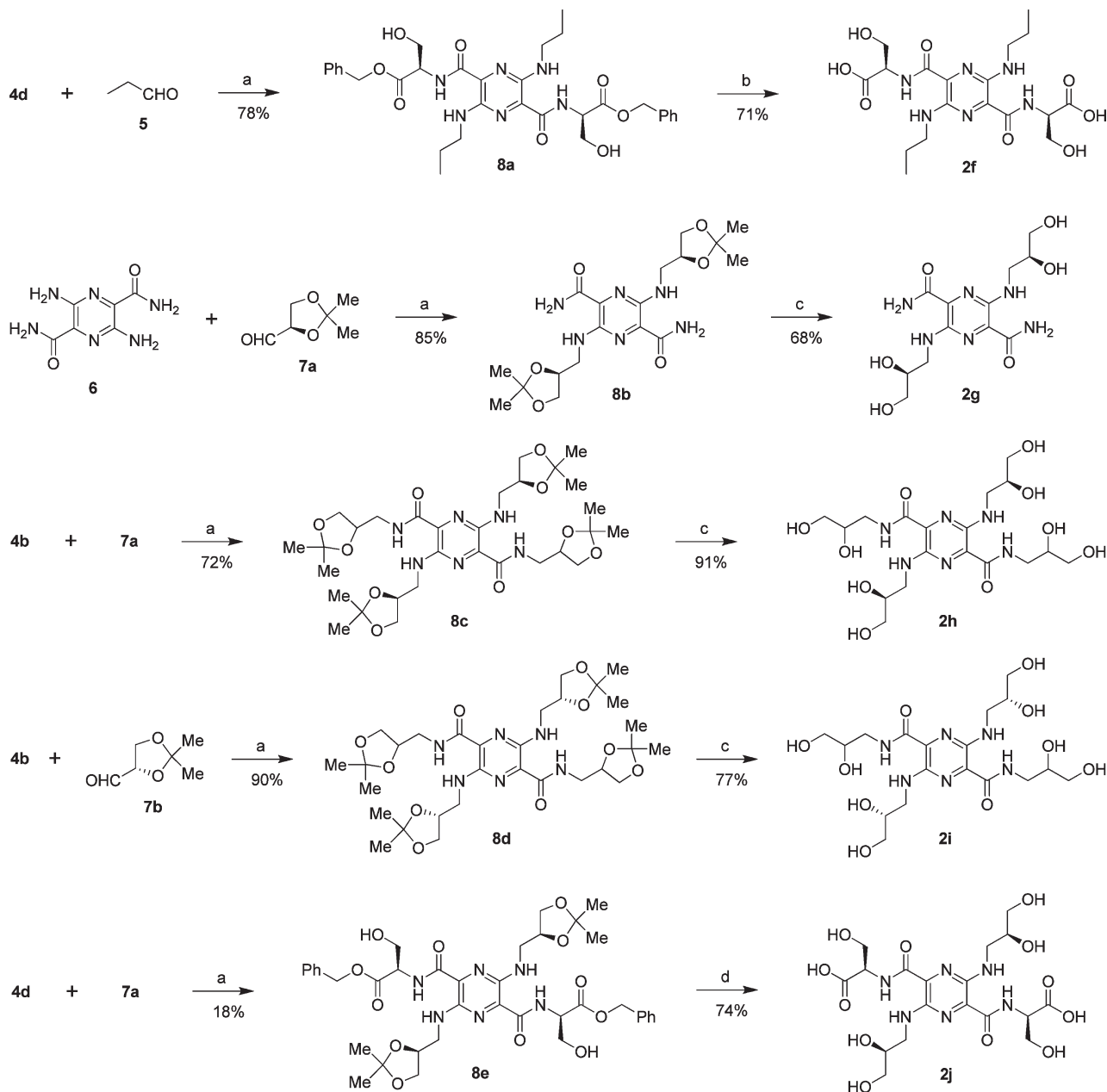
^a Reagents: (a) Et₃N, EDC·HCl, HOBt·H₂O, DMF; (b) 4 N HCl in dioxane; (c) EDC·HCl, HOBt·H₂O, DMF; (d) 1 N HCl, THF; (e) DIPEA, EDC·HCl, HOBt·H₂O, DMF; (f) H₂, 10% Pd-C, EtOH-H₂O (3:1, v/v); (g) HCO₂NH₄, 10% Pd-C, MeOH-H₂O (2:1, v/v), 60 °C. Note: the percentages shown under each arrow refer to reaction yields.

products. Consequently, a convenient and robust reductive amination method was developed for the synthesis of N-alkylated pyrazines,²² which was adopted for the preparation of the analogues **2f–j** (Scheme 2). The neutral N-alkylated pyrazine derivative **2g** was prepared by the reductive amination of D-glyceraldehyde acetonide (**7a**) with 3,6-diamino-2,5-dicarboxamide (**6**), followed by deprotection of the isopropylidene group with aqueous HCl. The bis(propyl) derivative **2f** was prepared by reductive amination of propionaldehyde with bis-benzyl ester **4d**, followed by subsequent transfer hydrogenation of the intermediate **8a**. The neutral **2h** and **2i** were made available by the initial reductive alkylation of the bis-acetonide **4b** with **7a** or L-glyceraldehyde acetonide (**7b**), respectively, followed by acidic hydrolysis of the acetonide groups. Finally, a similar reductive alkylation of **4d** with **7a** afforded **8e**, which was then treated with aqueous HCl to remove the acetonide groups followed by base hydrolysis of benzyl esters leading to the dianionic compound **2j**.

In Vitro Studies. Absorption (λ_{abs}) and emission (λ_{em}) maxima and plasma protein binding (PPB) were determined for all

compounds prior to in vivo evaluation (Table 1). Compounds **2a–e** exhibited absorption maxima in the range of 430–440 nm and emission maxima in the range of 550–560 nm. Compounds **2f–j** exhibited absorption maxima in the range of 480–490 nm and emission maxima in the range of 590–600 nm. The fluorescence quantum yield of **2c** in DMSO was measured to be 0.42. With the exception of **2f** and **2g**, all compounds exhibited low plasma protein binding (PPB), comparable to or lower (i.e., better) than iohalamate. As would be expected, the introduction of hydrophobic propyl group in **2f** resulted in high PPB. Surprisingly, the two regioisomeric compounds **2b** and **2g** exhibited measurably different PPB. It is possible that the primary carboxamide, which contains an additional site for hydrogen bonding at the amide site in **2g** is contributing to the higher interaction with the protein.

In Vivo Studies. All in vivo studies were performed in the Sprague–Dawley rat model. Plasma concentrations of the tracer agents were determined by HPLC at 0, 2, 6, and 24 h post administration of the injected dose. Urine was collected through

Scheme 2^a

^a Reagents: (a) $\text{Na}(\text{OAc})_3\text{BH}$, HOAc , DCE ; (b) HCO_2NH_4 , 10% $\text{Pd}-\text{C}$, $\text{MeOH}-\text{H}_2\text{O}$ (2:1, v/v), 60°C ; (c) 1 N HCl , THF ; (d) (i) c, (ii) 1 N NaOH . Note: the percentages shown under each arrow refer to reaction yields.

6 h post administration of the injected dose. Plasma clearance profiles and percent injected dose (%ID) recovered in the urine are given in Table 2. Among the neutral compounds, polyhydroxy derivatives **2h** and **2i** exhibit the highest %ID recovery from urine. Among the charged compounds, the dianionic serine derivatives **2d** and **2j** exhibit the highest %ID recovery from urine. The tetraanionic compound **2e** demonstrated the least % ID recovery from urine among the anionic compounds. This is consistent with the previous observation that optimal renal clearance is observed for those compounds having two negative charges, and that any increase in the charge adversely impacts renal clearance.^{24,25} Additionally, an HPLC analysis of the compounds **2c**, **2d**, **2h**, and **2j** prior to in vivo administration

and after recovery from urine did not reveal any appreciable amounts of degradation products. Because of the substantially lower amounts of **2b**, **2e**, and **2g** recovered in the urine with respect to the other pyrazine derivatives, plasma clearance studies of these derivatives were not pursued.

To ascertain whether any of the compounds had clearance affected by renal tubular secretion (a negative result being a necessary condition for pure GFR clearance), renal proximal tubule organic anion transport inhibition was next investigated. The in vivo clearance of four selected pyrazine derivatives was measured with and without probenecid (Figure 2). Iothalamate was included in this study as a comparator. In addition, ^{99m}Tc-MAG₃, a radiosynthetic imaging agent that is known to clear

via the tubular secretion pathway,^{19,25} was employed as a positive control.

Probenecid significantly decreased the clearance rate of ^{99m}Tc-MAG₃ ($p = 0.001$)²⁶ as expected from a non-GFR agent. Probenecid did not significantly affect the clearance rate of iothalamate ($p > 0.05$), also as expected from a GFR agent. Compounds **2h** and **2j** did not exhibit differences in clearance rates ($p > 0.05$). However, a statistically significant difference ($p < 0.05$) was detected in the clearance rate of the L-serine enantiomer **2c** with respect to the presence or absence of probenecid, whereas the D-serine enantiomer **2d** did not display a significant difference ($p > 0.05$). Therefore, renal tubular secretion is not a significant elimination pathway for compounds **2d**, **2h**, and **2j** in the rat.

Noninvasive real-time fluorescence monitoring of the clearance of compound **2h** is shown in Figure 3.²⁷ Each panel contains images of two mice. The mouse on the left was administered 300 μ L of a 2 mM solution (in PBS) of compound **2h**. The mouse on the right received 200 μ L of PBS only. The two administrations were given simultaneously. Compound **2h** is seen to distribute quickly throughout the body and then clears from the body and concentrates in the abdomen. Surgery after

the 60 min time point verified that the highly fluorescent spot in the abdomen was the bladder.

To follow the time dependence of the fluorescence quantitatively, an arbitrary region of interest (ROI) was drawn encompassing the area around the bladder and an approximately equal area was drawn near the shoulder on the series of optical images of the mouse injected with compound **2h** from 0 to 70 min. As a function of time, the fluorescence signal is seen to monotonically increase over the bladder region as expected (Figure 4). Over the shoulder region, this signal is seen to increase post administration of tracer agent and then wash out as a function of time as it is removed from the body by the renal system. This fluorescence decrease is consistent with the decrease in plasma concentration of **2h** as indicated in Table 2.

CONCLUSION

On the basis of the fluorescence properties, plasma protein binding data, the injected dose recovered in urine, the plasma clearance data, and the renal tubular secretion studies, the pyrazine serine derivatives **2d**, **2h**, and **2j** are viable candidates for translation to the clinic as exogenous fluorescent tracer agents for the measurement of GFR. In the rat animal model, these compounds display superior properties compared to iothalamate, which is currently an accepted standard for the measurement of GFR. Formal preclinical development studies are in progress and will be reported elsewhere.

EXPERIMENTAL SECTION

Chemistry. Unless otherwise noted, all solvents and reagents were used as supplied. Organic extracts were dried over either anhyd Na₂SO₄ or anhyd MgSO₄ and filtered using a fluted filter paper (P8) or a fritted glass funnel. Solvents were removed on a rotary evaporator under reduced pressure. Analytical TLC was performed on Analtech silica gel GF plates (250 μ m), and the components were visualized by UV light. Flash chromatography was carried out either using EMD silica gel 60 (40–63 μ m) or C18 silica gel (YMC ODS-A 120 Å I 25/40) in glass columns. Automated flash chromatography was carried out on a Teldyne Isco CombiFlash R_f system using prepacked silica gel columns. RP-LC/MS (ESI, positive ion mode) analyses were carried out on either a BDS Hypersil C18 3 μ m (50 mm $\times$ 4.6 mm) or a ThermoElectron Hypersil Gold C18 3 μ m (4.6 mm $\times$ 50 mm) column. Compounds were injected

Table 2. %ID Recovery in Urine, and Plasma Clearance Half-Lives^a

compd	%ID recovered in urine at 6 h	plasma half-life ^b (min)
2b	61 $\pm$ 3 (3) ^c	ND ^d
2c	82 $\pm$ 7 (3)	36 $\pm$ 6 (8)
2d	90 $\pm$ 1 (3)	29 $\pm$ 1 (6)
2e	61 $\pm$ 16 (3)	ND
2g	25 $\pm$ 4 (3)	ND
2h	88 $\pm$ 2 (3)	19 $\pm$ 1 (6)
2i	87 $\pm$ 4 (3)	ND
2j	85 $\pm$ 2 (3)	20 $\pm$ 1 (5)
iothalamate	80 $\pm$ 2 (6)	32 $\pm$ 2 (4)

^a The values are given as mean $\pm$ SEM (standard error of mean).

^b Terminal phase half-life from two compartment modeling. ^c Numbers in parentheses indicate number of test animals. ^d Not determined.

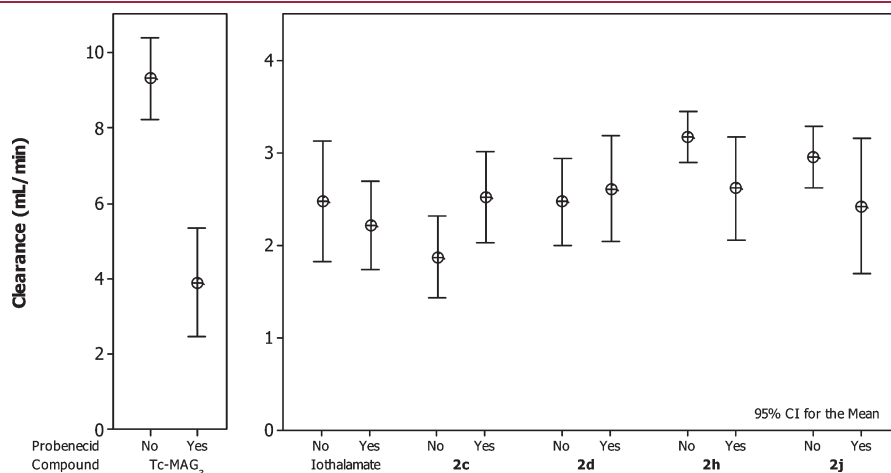


Figure 2. Clearance of ^{99m}Tc-MAG₃ with and without probenecid, and clearance of several pyrazine compounds and iothalamate comparator with and without probenecid.

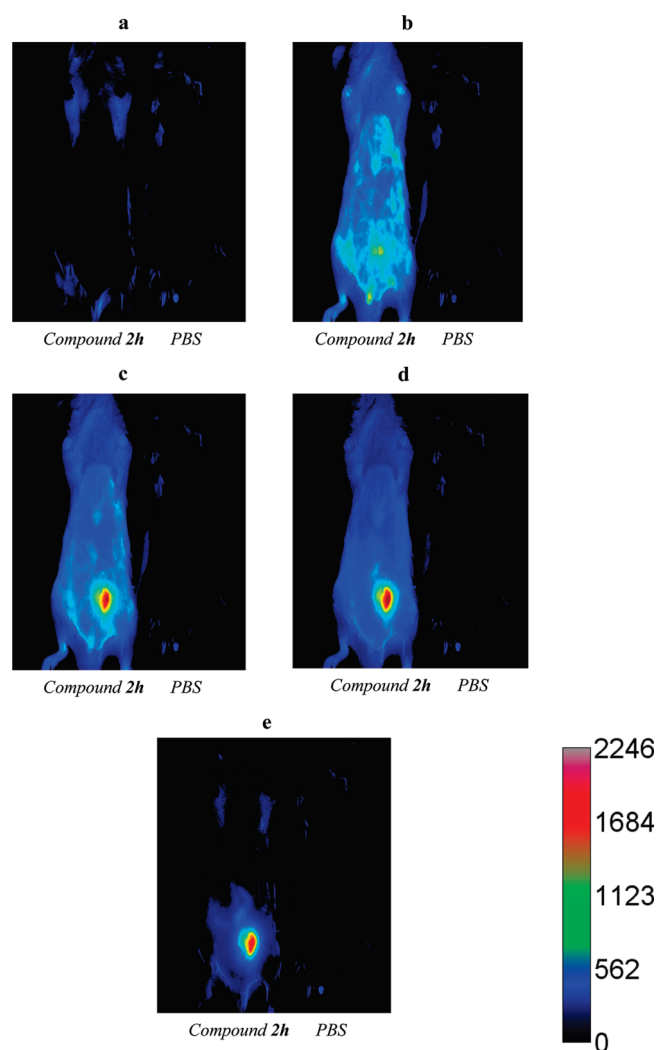


Figure 3. In vivo time-dependent fluorescence detection (with pixel intensity range bar graph). The mouse on the left was treated with compound **2h**, and the mouse on the right was treated with PBS; (a) preadministration, (b) 10 min post administration, (c) 20 min post administration, (d) 30 min post administration, (e) 60 min post administration. The ratio between most intense and least intense fluorescence in the 60 min post administration image was approximately a factor of 100.

using a gradient condition (5 to 50–95%B/6–10 min) with a flow rate of 1 mL/min (mobile phase A, 0.05% TFA in H₂O; mobile phase B, 0.05% TFA in CH₃CN). Preparative RP-HPLC was carried out using a Waters XBridge Prep C18 5 μ m OBD 30 mm $\times$ 150 mm or 19 $\times$ 250 mm column [λ_{max} , PDA (200–800 nm); flow, 50 mL/min; gradient, 5–50% B/10–17 min; mobile phase A, 0.1% TFA in H₂O; mobile phase B, 0.1% TFA in CH₃CN]. RP-HPLC analyses were carried out using a Phenomenex Luna 5 μ m C18(2) 100 $\text{\AA}$ 250 mm $\times$ 4.6 mm column [detection, UV; flow, 1 mL/min; gradient, 5/10% B to 50/90% B/20 min; mobile phase A, 0.1% TFA in H₂O; mobile phase B, 0.1% TFA in CH₃CN], and the chromatographic purities of all the compounds were >95%. UV/vis and fluorescence spectra were measured on a Shimadzu UV-3101 PC and Jobin Yvon Fluorolog-3 spectrometers, respectively. NMR spectra were recorded on either a Varian VNMR-500 spectrometer. ¹H Chemical shifts are expressed in parts per million (δ) relative to TMS (δ = 0) as an internal standard. ¹³C Chemical shifts are referenced to either TMS (δ = 0) or the residual solvent peaks in the

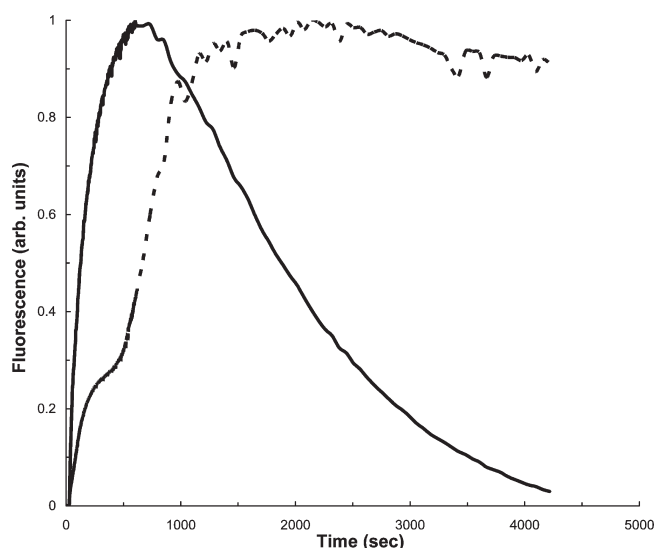


Figure 4. Fluorescence measured over the bladder (dashed line) and shoulder region (solid line) as a function of time (data was normalized to peak value of each ROI).

spectra. Coupling constants (*J*) are reported in Hz. HRMS (ESI) data was obtained on a ThermoFisher LTQ-Orbitrap mass spectrometer equipped with an IonMax electrospray ionization source in FTMS mode with resolution $\geq$ 30K. Elemental analyses were carried out by Atlantic Microlab, Inc., Norcross, GA.

3,6-Diamino-*N*²,*N*⁵-bis(2-*tert*-butoxyethyl)pyrazine-2,5-dicarboxamide (4a). A 100 mL round-bottom flask equipped with a magnetic stir bar was charged with diacid **1** (0.420 g, 2.10 mmol), 2-*tert*-butoxyethylamine $\cdot$ HCl (**3a**, 0.717 g, 4.66 mmol), EDC $\cdot$ HCl (0.894 g, 4.66 mmol), HOBt $\cdot$ H₂O (0.630 g, 4.66 mmol), and Et₃N (0.630 g, 6.36 mmol) in anhyd DMF (40 mL). The reaction mixture was stirred overnight at rt and concentrated in vacuo to $\sim$ 10 mL solution that was partitioned between EtOAc (125 mL) and satd NaHCO₃ (100 mL). The organic solution was washed with 10% aqueous citric acid (100 mL) and brine (50 mL). The solution was concentrated and purified via flash chromatography over silica gel using EtOAc–hexanes (2:1, v/v) as eluent to give **4a** (0.290 g, 35%). ¹H NMR (DMSO-*d*₆) δ 8.30 (t, *J* = 5.9 Hz, 2 H), 6.57 (s, 4H), 3.42 (dt, *J* = 5.3 Hz, 4 H), 3.36 (dt, *J* = 5.9 Hz, 4 H), 1.14 (s, 18 H). ¹³C NMR (DMSO-*d*₆) δ 164.8, 146.1, 126.1, 72.5, 59.7, 39.5, 27.2. HRMS (ESI) *m/z* calcd for C₁₈H₃₃N₆O₄ (*M* + H)⁺ 397.2558, found 397.2558; calcd for C₁₈H₃₂N₆O₄Na (*M* + Na)⁺ 419.2377, found 419.2376.

3,6-Diamino-*N*²,*N*⁵-bis(2-hydroxyethyl)pyrazine-2,5-dicarboxamide (2a). A 100 mL round-bottom flask equipped with a magnetic stir bar was charged with the diether **4a** (0.233 g, 0.588 mmol) and 4 N HCl–dioxane (8 mL) and stirred at rt overnight. The reaction was concentrated in vacuo, and the crude product ($\sim$ 0.170 g) was purified via RP-HPLC (5–50%B). The product containing fractions were concentrated in vacuo and vacuum-dried to afford **2a** (0.082 g, 49%) as an orange solid. ¹H NMR (DMSO-*d*₆) δ 8.30 (t, *J* = 5.9 Hz, 2H), 7.60 (s, 4 H), 4.83 (t, *J* = 5.9 Hz, 2 H), 3.45 (dt, *J* = 5.4 Hz, 4 H), 3.35 (dt, *J* = 5.9 Hz, 4 H). ¹³C NMR (DMSO-*d*₆) δ 165.4, 146.1, 126.7, 60.0, 41.8. HRMS (ESI) *m/z* calcd for C₁₀H₁₇N₆O₄ (*M* + H)⁺ 285.1306, found 285.1307; calcd for C₁₀H₁₆N₆O₄Na (*M* + Na)⁺ 307.1125, found 307.1126.

3,6-Diamino-*N*²,*N*⁵-bis[(2,2-dimethyl-1,3-dioxolan-4-yl)methyl]pyrazine-2,5-dicarboxamide (4b). A mixture of diacid **1** (0.350 g, 1.77 mmol), 2,2-dimethyl-1,3-dioxolane-4-methanamine (**3b**, 0.933 mL, 7.20 mmol), HOBt $\cdot$ H₂O (0.812 g, 5.30 mmol), and EDC $\cdot$ HCl (1.02 g, 5.32 mmol) were stirred together in DMF (20 mL) for 16 h

at rt. The mixture was concentrated to dryness, and the residue was partitioned between EtOAc and water. The layers were separated, and the EtOAc solution was washed with satd NaHCO_3 and brine. The EtOAc solution was concentrated to afford the diastereomeric bis-amide **4b** (0.665 g, 88%) as a yellow solid. ^1H NMR (CDCl_3) δ 8.38 (t, J = 5.8 Hz, 2 H), 6.55 (s, 4 H), 4.21 (quintet, J = 5.8 Hz, 2 H), 3.98 (dd, J = 8.4 Hz, 6.3 Hz, 2 H), 3.65 (dd, J = 8.4 Hz, J = 5.8 Hz, 2 H), 3.39 (apparent quartet—diastereotopic mixture, J = 5.9 Hz, 4 H), 1.35 (s, 6 H), 1.26 (s, 6 H). ^{13}C NMR (CDCl_3) δ 165.7, 146.8, 126.8, 109.2, 74.8, 67.2, 42.2, 41.1, 27.6, 26.1. RP-LC/MS (ESI) m/z 425.4 ($\text{M} + \text{H}$) $^+$ (t_R = 4.00 min, 5–95%B/6 min). HRMS (ESI) m/z calcd for $\text{C}_{18}\text{H}_{28}\text{N}_6\text{O}_6\text{Na}$ ($\text{M} + \text{Na}$) $^+$ 447.1963, found 447.1960. Anal. Calcd for $\text{C}_{18}\text{H}_{28}\text{N}_6\text{O}_6$: C, 50.93; H, 6.65; N, 19.80. Found: C, 51.35; H, 7.12; N, 19.59.

3,6-Diamino- N^2,N^5 -bis(2,3-dihydroxypropyl)pyrazine-2,5-dicarboxamide (2b). The above bis-amide **4b** was dissolved in THF (100 mL) and treated with 1.0 N HCl (2 mL). After hydrolysis was complete, the mixture was treated with K_2CO_3 (1 g) and stirred for 1 h and filtered through a plug of C18 silica gel using methanol. The filtrate was concentrated to dryness and the residue was triturated with MeOH (50 mL). The solids were filtered and discarded, and the residue was treated with ether (50 mL). The precipitate was collected by filtration and dried under high vacuum. This material was purified by radial flash chromatography using CHCl_3 –MeOH (19:1 to 1:1, v/v) to afford **2b** (0.221 g, 36%) as an orange solid. ^1H NMR ($\text{DMSO}-d_6$) δ 8.00 (br m, 6 H), 5.39 (br s, 2 H), 4.88 (br s, 2 H), 3.63–3.71 (complex m, 2 H), 3.40 (dd, J = 11.1, 5.1 Hz, 2 H), 3.28 (dd, J = 11.1, 6.6 Hz, 2 H), 2.92 (dd, J = 12.6, 3.3 Hz, 2 H), 2.65 (dd, J = 12.6, 8.4 Hz, 2 H). RP-LC/MS (ESI) m/z 345 ($\text{M} + \text{H}$) $^+$ (t_R = 4.13 min, 5–95% gradient acetonitrile in 0.1% TFA over 10 min on a 30 mm column). HRMS (ESI) m/z calcd for $\text{C}_{12}\text{H}_{20}\text{N}_6\text{O}_6\text{Na}$ ($\text{M} + \text{Na}$) $^+$ 367.1337, found 367.1336.

3,6-Diamino- N^2,N^5 -bis[(S)-1-(benzyloxy)-3-hydroxy-1-oxopropan-2-yl]pyrazine-2,5-dicarboxamide (4c). A 250 mL round-bottom flask equipped with a Claisen adapter and an addition funnel was charged with L-serine benzyl ester hydrochloride (**3c**, 4.87 g, 21.0 mmol) and anhydrous DMF (100 mL) was cannulated into it. The resulting colorless solution was cooled in an ice-bath and stirred for 15 min under N_2 atmosphere. Then DIPEA (3.83 mL, 22.0 mmol) was added dropwise via addition funnel over a 30 min period, and after 30 min, the cooling bath was removed and diacid **1** (1.98 g, 10.0 mmol) was added in one portion. The brick-red suspension was allowed to stir for 30 min before the addition of $\text{HOBt} \cdot \text{H}_2\text{O}$ (3.37 g, 22.0 mmol) in one portion. After another 15 min, the reaction flask was once again cooled in an ice-bath, and EDC $\cdot$ HCl (4.22 g, 22.0 mmol) was added in portions over a 15 min period. The resulting somewhat lighter and brown suspension was slowly allowed to warm to rt and stirred overnight (ca. 17 h) under N_2 . The reaction mixture was diluted with EtOAc (500 mL) and transferred to a 1 L separatory funnel. The solution was washed with milli-Q H_2O (150 mL), and the aq phase was further extracted with EtOAc (100 mL). The combined organic extracts were successively washed with 150 mL portions of water, 0.50 M KHSO_4 , water, satd NaHCO_3 ($\times 2$), water, and brine. Removal of the solvent followed by drying under high vacuum gave 4.75 g of the crude product as an orange fluffy solid, which upon flash chromatography over silica gel using CHCl_3 –MeOH (19:1, v/v) as eluant (sample adsorbed on silica gel was loaded on the column), afforded the bis-amide **4c** (4.34 g, 79%) as an orange solid: R_f 0.40 [CHCl_3 –MeOH (9:1, v/v)]. ^1H NMR ($\text{DMSO}-d_6$) δ 8.56 (d, J = 8.3 Hz, 2 H, exchangeable with D_2O), 7.40–7.31 (m, 10 H), 6.76 (s, 4 H, exchangeable with D_2O), 5.37 (t, J = 5.6 Hz, 2 H), 5.20 (distorted AB pair, 4 H), 4.67–4.64 (dt, J = 8.3, 3.8 Hz, 2 H), 3.97–3.93 (m, 2 H), 3.81–3.77 (m, 2 H). ^{13}C NMR ($\text{DMSO}-d_6$) δ 170.1, 164.9, 146.4, 135.8, 128.4, 128.0, 127.6, 125.9, 66.2, 61.1, 54.4. RP-LC/MS (ESI) m/z 553.3 ($\text{M} + \text{H}$) $^+$ (t_R = 4.24 min, 15–95%B/6 min). Anal. Calcd for $\text{C}_{26}\text{H}_{28}\text{N}_6\text{O}_8$: C, 56.52; H, 5.11; N, 15.21. Found: C, 56.53; H, 5.05; N, 15.16.

3,6-Diamino- N^2,N^5 -bis[(S)-1,3-dihydroxy-1-oxopropan-2-yl]pyrazine-2,5-dicarboxamide (2c). The bis-amide **4c** (3.87 g, 7.00 mmol) was weighed into a 500 mL round-bottom flask equipped with a Claisen adapter and anhyd EtOH (225 mL), and milli-Q H_2O (50 mL) were added. The resulting orange suspension (partially soluble) was briefly sonicated and 10% Pd–C (0.39 g) was added as a slurry in H_2O (25 mL). The reaction mixture was thoroughly purged first with argon and then with H_2 and stirred under H_2 atmosphere (slow bubbling) at rt for 5.5 h. The reaction mixture was once again purged with Ar, and the catalyst was removed by filtration over a Celite bed. The bed was thoroughly washed with EtOH– H_2O (400 mL; 1:1, v/v), and the combined filtrates were concentrated in vacuo and further dried under high vacuum. The resulting orange residue (2.45 g) was triturated with CH_3CN (100 mL; sonicated briefly to get the material off the walls of the flask) to give **2c** (2.29 g, 88%) as a red powder. ^1H NMR ($\text{DMSO}-d_6$) δ 8.46 (d, J = 8.3 Hz, 2 H, exchangeable with D_2O), 6.78 (br s, 4 H, exchangeable with D_2O), 4.48–4.45 (dt, J = 8.3, 3.9 Hz, 2 H; collapses to a triplet at δ = 4.47 ppm with J = 3.7 Hz upon D_2O exchange), 3.88 (dd, J = 10.9, 3.8 Hz, 2 H), 3.74 (dd, J = 10.9, 3.6 Hz, 2 H). ^{13}C NMR ($\text{DMSO}-d_6$) δ 171.5, 164.6, 146.3, 125.9, 61.1, 54.2. RP-LC/MS (ESI) m/z 373.3 ($\text{M} + \text{H}$) $^+$ (t_R = 2.62 min, 5–95%B/6 min). Anal. Calcd for $\text{C}_{12}\text{H}_{16}\text{N}_6\text{O}_8$: C, 38.71; H, 4.33; N, 22.57. Found: C, 38.67; H, 4.50; N, 22.27.

3,6-Diamino- N^2,N^5 -bis[(R)-1-(benzyloxy)-3-hydroxy-1-oxopropan-2-yl]pyrazine-2,5-dicarboxamide (4d). The reaction of diacid **1** (9.91 g, 50.0 mmol) with D-serine benzyl ester hydrochloride (**3d**, 24.33 g, 105.0 mmol) in the presence of DIPEA (19.16 mL, 110.0 mmol), $\text{HOBt} \cdot \text{H}_2\text{O}$ (17.61 g, 115.0 mmol), and EDC $\cdot$ HCl (22.05 g, 115.0 mmol) in DMF (300 mL) was carried out overnight (ca. 14 h) as described for the preparation of **4c**. The dark solution was concentrated to a syrupy residue under high vacuum (bath temp 60 $^\circ\text{C}$) that was partitioned between EtOAc and milli-Q H_2O (400 mL each). The layers were separated and the aqueous layer was further extracted with EtOAc (3×200 mL), and the combined EtOAc extracts were successively washed with 0.50 M KHSO_4 , saturated NaHCO_3 , H_2O , and brine (250 mL each). Removal of the solvent left an orange slush that was dried under high vacuum overnight to give 23.7 g of an orange solid. The crude product was subjected to flash chromatography over silica gel using CHCl_3 to CHCl_3 –MeOH (97:3, v/v) as eluant in a gradient fashion (sample adsorbed on silica gel was loaded on the column; carried out in 4 runs) to give the bis-amide **4d** (19.6 g, 71%) as an orange solid: R_f 0.45 [CHCl_3 –MeOH (9:1, v/v)]. ^1H NMR ($\text{DMSO}-d_6$) δ 8.56 (d, J = 8.0 Hz, 2 H, exchangeable with D_2O), 7.40–7.33 (m, 10 H), 6.76 (s, 4 H, exchangeable with D_2O), 5.37 (t, J = 5.5 Hz, 2 H), 5.20 (distorted AB pair, 4 H), 4.66–4.63 (dt, J = 8.0, 4.0 Hz, 2 H), 3.97–3.93 (m, 2 H), 3.81–3.77 (m, 2 H). ^{13}C NMR ($\text{DMSO}-d_6$) δ 170.1, 164.9, 146.4, 135.8, 128.4, 128.0, 127.6, 125.9, 66.2, 61.1, 54.4. RP-LC/MS (ESI) m/z 553.3 ($\text{M} + \text{H}$) $^+$ (t_R = 4.44 min, 5–95%B/6 min). Anal. Calcd for $\text{C}_{26}\text{H}_{28}\text{N}_6\text{O}_8$: C, 56.52; H, 5.11; N, 15.21. Found: C, 56.39; H, 5.11; N, 14.99.

3,6-Diamino- N^2,N^5 -bis[(R)-1,3-dihydroxy-1-oxopropan-2-yl]pyrazine-2,5-dicarboxamide (2d). The bis-amide **4d** (7.74 g, 14.0 mmol) was hydrogenated in the presence of 10% Pd–C (0.774 g) in EtOH– H_2O (560 mL; 3:1, v/v) for 5 h as described for the preparation of **2c**. After a similar work up and isolation procedure, **2d** (4.89 g, 94%) was obtained as an orange powder. ^1H NMR ($\text{DMSO}-d_6$) δ 8.46 (d, J = 8.3 Hz, 2 H, exchangeable with D_2O), 6.78 (br s, 4 H, exchangeable with D_2O), 4.48–4.45 (dt, J = 8.1, 3.9 Hz, 2 H; collapses to a triplet at δ = 4.47 ppm with J = 3.7 Hz upon D_2O exchange), 3.88 (dd, J = 11.1, 3.9 Hz, 2 H), 3.74 (dd, J = 11.1, 3.7 Hz, 2 H). ^{13}C NMR ($\text{DMSO}-d_6$) δ 171.6, 164.7, 146.4, 125.9, 61.2, 54.3. RP-LC/MS (ESI) m/z 373.2 ($\text{M} + \text{H}$) $^+$ (t_R = 2.86 min, 5–95%B/6 min). Anal. Calcd for $\text{C}_{12}\text{H}_{16}\text{N}_6\text{O}_8$: C, 38.71; H, 4.33; N, 22.57. Found: C, 38.44; H, 4.51; N, 22.33.

3,6-Diamino-*N*²,*N*⁵-bis[(*R*)-1,2-(dibenzoyloxycarbonyl)ethyl]pyrazine-2,5-dicarboxamide (4e). A mixture of sodium salt of diacid **1** (0.600 g, 2.48 mmol), *D*-Asp(OBn)-OBn-*p*-TsOH salt (**3e**, 2.43 g, 5.00 mmol), HOBT·H₂O (0.919 g, 6.00 mmol) and EDC·HCl (1.14 g, 5.95 mmol) in DMF (50 mL) was treated with Et₃N (4 mL). The resulting mixture was stirred overnight at rt. The reaction mixture was concentrated, and the residue was partitioned between water and EtOAc. The EtOAc layer was separated and washed successively with satd sodium bicarbonate, water, and brine. The EtOAc solution was concentrated, and the residue was purified by flash chromatography [SiO₂, CHCl₃–MeOH (50/1 to 10/1, v/v)] to afford the bis-amide **4e** (1.15 g, 58% yield) as a yellow foam. ¹H NMR (CDCl₃) δ 8.61 (d, *J* = 8.4 Hz, 2 H), 7.29–7.39 (m, 20 H), 5.85 (br s, 4 H), 5.22 (ABq, *J* = 10.0 Hz, Δ*ν*_{AB} = 17.3 Hz, 4 H), 5.10 (ABq, *J* = 12.2 Hz, Δ*ν*_{AB} = 34.3 Hz, 4 H), 5.06–5.09 (m, 2 H), 3.11 (ABq of d, *J* = 17.0, 5.1 Hz, Δ*ν*_{AB} = 77.9 Hz, 4 H). ¹³C NMR (CDCl₃) δ 170.7, 170.7, 165.4, 147.0, 135.7, 135.6, 129.0, 128.9, 128.8, 128.75, 128.7, 126.9, 68.0, 67.3, 49.1, 37.0. RP-LC/MS (ESI) *m/z* 789 (M + H)⁺ (*t*_R = 5.97 min, 5–95% gradient acetonitrile in 0.1% TFA over 10 min on a 250 mm column).

3,6-Diamino-*N*²,*N*⁵-bis[(*R*)-1,2-(dicarboxy)ethyl]pyrazine-2,5-dicarboxamide (2e). To a well stirred solution of tetra-benzyl ester **4e** (0.510 g, 0.650 mmol) in THF (20 mL) and water (10 mL) were added 10% Pd/C (0.50 g) and ammonium formate (1.00 g, 15.9 mmol). The resulting mixture was heated to 60 °C for 2 h and allowed to cool to rt. The mixture was filtered through Celite and concentrated. The resulting material was purified by reverse phase medium pressure chromatography (C18, 10–70% manual gradient acetonitrile in 0.1% TFA) to afford **2e** (0.138 g, 54%) as an orange solid. ¹H NMR (DMSO-*d*₆) δ 8.62 (d, *J* = 8.4 Hz, 2 H), 6.67 (br s, 4 H), 4.73 (dt, *J* = 8.4, 5.4 Hz, 2 H), 2.74–2.88 (complex m, 4 H). ¹³C NMR (DMSO-*d*₆) δ 172.6, 165.2, 147.0, 126.6, 60.8, 49.1. RP-LC/MS (ESI) *m/z* 429 (M + H)⁺ (*t*_R = 4.01 min, 5–95% gradient acetonitrile in 0.1% TFA over 10 min on a 250 mm column). HRMS (ESI) *m/z* calcd for C₁₄H₁₄N₆O₁₀ (M – 2 H)^{2–} 213.0391, found 213.0388.

***N*²,*N*⁵-Bis[(*R*)-1-(benzyloxy)-3-hydroxy-1-oxopropan-2-yl]-3,6-bis(propylamino)pyrazine-2,5-dicarboxamide (8a).** To a solution of the bis-benzyl ester **4d** (0.890 g, 1.61 mmol) and propionaldehyde (**5**, 0.460 mL, 6.44 mmol) in anhyd DCE (65 mL) was added HOAc (0.368 mL, 6.44 mmol) with stirring at 0 °C under argon atmosphere. Then Na(OAc)₃BH (1.36 g, 6.44 mmol) was introduced in small portions and the reddish suspension was slowly allowed to warm to rt and stirred overnight (ca. 24 h) under argon. The reaction was quenched by a slow addition of satd NaHCO₃ at 0 °C and extracted with CHCl₃ (150 mL). The CHCl₃ extracts were successively washed with H₂O and brine (50 mL each). Removal of the solvent gave 1.04 g of a red solid, which upon flash chromatography over silica gel [CHCl₃–MeOH (39:1, v/v)] afforded **8a** (0.800 g, 78%) as a dark-red solid. ¹H NMR (CDCl₃) δ 8.68 (d, *J* = 7.4 Hz, 2 H), 7.60 (t, *J* = 5.6 Hz, 2 H), 7.37–7.33 (m, 10 H), 5.26 (s, 4 H), 4.78–4.75 (dt, *J* = 7.3, 3.7 Hz, 2 H), 4.08 (dd, *J* = 6.1, 3.9 Hz, 4 H), 3.39–3.28 (m, 4 H), 2.64 (t, *J* = 6.2 Hz, 2 H), 1.65–1.61 (m, 4 H), 0.97 (t, *J* = 7.4 Hz, 6 H). ¹³C NMR (CDCl₃) δ 169.9, 166.5, 146.0, 135.1, 128.7, 128.6, 128.2, 125.8, 67.6, 63.6, 55.1, 42.9, 22.7, 11.7. RP-LC/MS (ESI) *m/z* 637.4 (M + H)⁺ (*t*_R = 5.13 min, 5–95% B/6 min). Anal. Calcd for C₃₂H₄₀N₆O₈: C, 60.37; H, 6.33; N, 13.20. Found: C, 60.49; H, 6.42; N, 13.13.

***N*²,*N*⁵-Bis[(*R*)-1,3-dihydroxy-1-oxopropan-2-yl]-3,6-bis(propylamino)pyrazine-2,5-dicarboxamide (2f).** A solution of the bis-benzyl ester **8a** (0.700 g, 1.10 mmol) in MeOH (150 mL) was purged with argon and a slurry of 10% Pd/C (0.350 g) in water (8 mL) was added. Then ammonium formate (0.480 g, 7.61 mmol) was added, and the resulting mixture was heated to 60 °C and stirred for 1 h. The reaction mixture was allowed to cool to room temperature, filtered through Celite, and concentrated on a rotary evaporator. The crude product (0.559 g) was subjected to purification by preparative RP-HPLC

(Sunfire Prep C18 OBD 5 μm 50 mm × 10 mm, 5–95% B/13 min) to give **2f** (0.356 g, 71%) as dark-red powder. ¹H NMR (DMSO-*d*₆) δ 8.54 (d, *J* = 7.8 Hz, 2 H), 4.94–4.87 (m, 4 H), 4.77 (dd, *J* = 10.8, 5.8 Hz, 2 H), 4.45–4.42 (dt, *J* = 8.1, 3.8 Hz, 2 H), 3.89 (dd, *J* = 11.0, 3.7 Hz, 2 H), 3.79 (dd, *J* = 11.0, 3.4 Hz, 2 H), 3.40–3.31 (m, 4 H), 1.61–1.56 (m, 4 H), 0.94 (t, *J* = 7.4 Hz, 6 H). ¹³C NMR (DMSO-*d*₆) δ 172.0, 165.5, 145.9, 126.1, 61.4, 54.7, 42.7, 22.8, 11.9. RP-LC/MS (ESI) *m/z* 457.4 (M + H)⁺ (*t*_R = 3.77 min, 5–95% B/6 min). HRMS (ESI) *m/z* calcd for C₁₈H₂₉N₆O₈ (M + H)⁺ 457.2041, found 457.2044.

3,6-Bis{[(*S*)-2,2-dimethyl-1,3-dioxolan-4-yl]methylamino}-pyrazine-2,5-dicarboxamide (8b). The reaction of 3,6-diaminopyrazine-2,5-dicarboxamide (**6**, 0.196 g, 1.00 mmol) with (*R*)-(+)-2,2-dimethyl-1,3-dioxolane-4-carbaldehyde (**7a**, 0.390 g, 3.00 mmol) in the presence of HOAc (0.180 g, 3.00 mmol) and Na(OAc)₃BH (0.633 g, 3.00 mmol) in anhyd DCE (10 mL) was carried out overnight as described for the preparation of **8a**. A similar work up afforded **8b** (0.36 g, 85%) as a red solid, which was used without further purification. ¹H NMR (CDCl₃) δ 7.95 (br t, 2 H), 7.62 (br, 2 H), 5.60 (br, 2 H), 4.37 (m, 21 H), 4.12 (m, 2 H), 3.81 (m, 2 H), 3.58 (m, 4 H), 1.55 (s, 6 H), 1.38 (s, 6 H). ¹³C NMR (CDCl₃) δ 168.1, 146.1, 126.1, 109.4, 74.9, 67.3, 43.4, 27.1, 25.5. RP-LC/MS (ESI) *m/z* 425.4 (M + H)⁺.

3,6-Bis[(*S*)-2,3-dihydroxypropylamino]pyrazine-2,5-dicarboxamide (2g). The bis-acetonide **8b** (0.36 g, 0.85 mmol) was dissolved in THF (5 mL) and treated with 1 N HCl (1 mL). The reaction mixture was stirred overnight, neutralized with 1 N NaOH (1 mL), and evaporated to dryness. The crude product was subjected to flash chromatography over C18 silica gel using water as eluent to afford **2g** (0.200 g, 68%). ¹H NMR (DMSO-*d*₆) δ 7.91 (br t, 2 H), 7.60 (br, 2 H), 4.75 (br, 2 H), 4.60 (m, 2 H), 3.60 (m, 4 H), 3.20 (m, 4 H). ¹³C NMR (DMSO-*d*₆) δ 167.9, 145.5, 125.8, 70.4, 63.8, 43.6. HRMS (ESI) *m/z* calcd for C₁₂H₂₁N₆O₆ (M + H)⁺ 345.1517, found 345.1457.

***N*²,*N*⁵-Bis[(2,2-dimethyl-1,3-dioxolan-4-yl)methyl]-3,6-bis{[(*S*)-2,2-dimethyl-1,3-dioxolan-4-yl]methylamino}pyrazine-2,5-dicarboxamide (8c).** The reaction of bis-acetonide **4b** (1.72 g, 4.05 mmol) with aldehyde **7a** (2.64 g, 20.3 mmol) in the presence of HOAc (1.13 mL, 19.6 mmol) and Na(OAc)₃BH (4.18 g, 19.7 mmol) in anhyd DCE (50 mL) was carried out overnight (ca. 22 h) as described for the preparation of **8a**. The reaction was found to be incomplete (RP-LC/MS) at this stage and subsequently treated with additional aldehyde **7a** (0.70 g, 5.38 mmol), HOAc (0.300 mL, 5.20 mmol), and Na(OAc)₃BH (1.10 g, 5.19 mmol) and continued overnight. After a similar work up described in the preparation of **8a**, the crude product (4.30 g) obtained was subjected to automated flash chromatography [column: RediSep R_f 330 g silica gel; λ_{max} 280 nm; flow, 80 mL/min; gradient, 0–2% B/40 min (mobile phase A, CHCl₃; mobile phase B, MeOH)] to afford **8c** (1.90 g, 72%) as an orange powder. ¹H NMR (CDCl₃) δ 8.10 (t, *J* = 6.0 Hz, 2 H), 8.06 (t, *J* = 6.0 Hz, 2 H), 4.36–4.31 (m, 4 H), 4.09–4.06 (m, 4 H), 3.79–3.76 (m, 2 H), 3.74–3.67 (m, 2 H), 3.66–3.52 (m, 8 H), 1.49 (s, 3 H), 1.48 (s, 3 H), 1.45 (s, 6 H), 1.37 (s, 6 H), 1.36 (s, 6 H). RP-LC/MS (ESI) *m/z* 653.3 (M + H)⁺ (*t*_R = 4.83 min, 5–95% B/6 min). Anal. Calcd for C₃₀H₄₈N₆O₁₀: C, 55.20; H, 7.41; N, 12.88. Found: C, 55.14; H, 7.37; N, 12.60.

***N*²,*N*⁵-Bis(2,3-dihydroxypropyl)-3,6-bis[(*S*)-2,3-dihydroxypropylamino]pyrazine-2,5-dicarboxamide (2h).** To an orange solution of tetra-acetonide **8c** (3.68 g, 5.64 mmol) in THF (50 mL) was added 1.0 N HCl solution (45 mL) and stirred for 2 h at rt in an atmosphere of argon. Most of the THF was removed; the aq mixture was neutralized with 1.0 N NaOH, and evaporated to dryness. The residue thus obtained was further dried under high vacuum to give the crude product (5.86 g), which upon purification by gravity chromatography over C18 silica gel (gradient elution with water to 35% MeOH in water), afforded **2h** (2.52 g, 91%) as a red powder. ¹H NMR (DMSO-*d*₆) δ 8.50 (t, *J* = 5.9 Hz, 2 H, exchangeable with D₂O), 7.99 (t, *J* = 5.7 Hz, 2 H,

exchangeable with D₂O), 4.91 (dd, $J = 4.9, 1.3$ Hz, 2 H, exchangeable with D₂O), 4.81 (d, $J = 4.9$ Hz, 2 H, exchangeable with D₂O), 4.69–4.63 (m, 4 H, exchangeable with D₂O), 3.66–3.56 (m, 6 H), 3.47–3.31 (m, 10 H), 3.28–3.19 (m, 4 H). ¹³C NMR (DMSO-*d*₆) δ 165.9, 145.9, 126.4, 70.8, 70.41, 70.36, 64.6, 64.31, 64.29, 44.15, 44.13, 42.9. RP-HPLC (254 nm) 97.3% ($t_R = 11.76$ min, 5–50% B/20 min). RP-LC/MS (ESI) m/z 493.4 (M + H)⁺ ($t_R = 3.00$ min, 5–95% B/6 min). HRMS (ESI) m/z calcd for C₁₈H₃₂N₆O₁₀Na (M + Na)⁺ 515.2072, found 515.2059. Anal. Calcd for C₁₈H₃₂N₆O₁₀·H₂O: C, 42.35; H, 6.71; N, 16.46. Found: C, 42.31; H, 6.58; N, 16.43.

N²,N⁵-Bis[(2,2-dimethyl-1,3-dioxolan-4-yl)methyl]-3,6-bis{[(R)-2,2-dimethyl-1,3-dioxolan-4-yl]methylamino}pyrazine-2,5-dicarboxamide (8d). The reaction of bis-acetonide 4b (1.00 g, 2.36 mmol) with (S)-2,2-dimethyl-1,3-dioxolane-4-carbaldehyde (7b, 0.920 g, 7.07 mmol) in the presence of HOAc (0.400 mL, 6.94 mmol) and Na(OAc)₃BH (1.46 g, 6.89 mmol) in anhyd DCE (40 mL) was carried out overnight (ca. 22 h) as described for the preparation of 8c [including further treatment with additional reagents aldehyde 7b (0.920 g, 7.07 mmol), HOAc (0.400 mL, 6.94 mmol), and Na(OAc)₃BH (1.46 g, 6.89 mmol) overnight]. After a similar work up, the crude product (1.45 g) was subjected to an automated flash chromatography [column: RediSep R_f 120 g silica gel; λ_{max} 280 nm; flow, 40 mL/min; gradient, 0–2% B/40 min, 2% B/90 min (mobile phase A, CHCl₃; mobile phase B, MeOH)] to afford 8d (1.38 g, 90%) as an orange–red powder. ¹H NMR (CDCl₃) δ 8.10 (t, $J = 6.0$ Hz, 2 H), 8.06 (t, $J = 6.0$ Hz, 2 H), 4.36–4.31 (m, 4 H), 4.09–4.06 (m, 4 H), 3.79–3.76 (m, 2 H), 3.74–3.70 (m, 2 H), 3.66–3.52 (m, 8 H), 1.49 (s, 3 H), 1.48 (s, 3 H), 1.45 (s, 6 H), 1.37 (s, 6 H), 1.36 (s, 6 H). RP-LC/MS (ESI) m/z 653.5 (M + H)⁺ ($t_R = 4.83$ min, 5–95% B/6 min). Anal. Calcd for C₃₀H₄₈N₆O₁₀: C, 55.20; H, 7.41; N, 12.88. Found: C, 54.98; H, 7.50; N, 12.73.

N²,N⁵-Bis(2,3-dihydroxypropyl)-3,6-bis[(R)-2,3-dihydroxypropylamino]pyrazine-2,5-dicarboxamide (2i). The deprotection of tetra-acetonide 8d (1.00 g, 1.53 mmol) with 1.0 N HCl (15 mL) in THF (25 mL) was carried out for 5 h as described for the preparation of 2h. After a similar work up, the crude product (2.01 g) was subjected to purification by gravity chromatography over C18 silica gel (gradient elution with water to 25% MeOH in water) to give 2i (0.58 g, 77%) as an orange powder. ¹H NMR (DMSO-*d*₆) δ 8.50 (t, $J = 5.8$ Hz, 2 H, exchangeable with D₂O), 7.99 (t, $J = 5.4$ Hz, 2 H, exchangeable with D₂O), 4.91 (d, $J = 3.4$ Hz, 2 H), 4.80 (d, $J = 4.7$ Hz, 2 H, exchangeable with D₂O), 4.68–4.63 (m, 4 H, exchangeable with D₂O), 3.63–3.55 (m, 6 H), 3.47–3.31 (m, 10 H), 3.27–3.19 (m, 4 H). ¹³C NMR (DMSO-*d*₆) δ 165.9, 145.94, 145.93, 126.4, 70.8, 70.41, 70.36, 64.7, 64.31, 64.29, 44.14, 44.12, 42.9. RP-HPLC (254 nm) 98.2% ($t_R = 11.78$ min, 5–50% B/20 min). RP-LC/MS (ESI) m/z 493.3 (M + H)⁺ ($t_R = 2.97$ min, 5–95% B/6 min). HRMS (ESI) m/z calcd for C₁₈H₃₂N₆O₁₀Na (M + Na)⁺ 515.2072, found 515.2069. Anal. Calcd for C₁₈H₃₂N₆O₁₀·H₂O: C, 42.35; H, 6.71; N, 16.46. Found: C, 42.75; H, 6.94; N, 16.53.

N²,N⁵-Bis[(R)-1-(benzyloxy)-3-hydroxy-1-oxopropan-2-yl]-3,6-bis{[(S)-2,2-dimethyl-1,3-dioxolan-4-yl]methylamino}pyrazine-2,5-dicarboxamide (8e). The reaction of bis-benzyl ester 4d (0.50 g, 0.905 mmol) with aldehyde 7a (0.71 g, 5.43 mmol) in the presence of HOAc (0.31 mL, 5.43 mmol) and Na(OAc)₃BH (1.15 g, 5.43 mmol) in anhyd DCE (20 mL) was carried out overnight as described for the preparation of 8c [including further treatment with additional reagents aldehyde 7a (0.71 g, 5.43 mmol), HOAc (0.71 g, 5.43 mmol), and Na(OAc)₃BH (1.15 g, 5.43 mmol) overnight]. After a similar work up, the crude product (0.80 g) was subjected to an automated flash chromatography [column: RediSep R_f 40 g silica gel; λ_{max} 280 nm; flow, 15 mL/min; gradient, 0–3% B/30 min, 3% B/50 min (mobile phase A, CHCl₃; mobile phase B, MeOH)] to afford 8e (0.13 g, 18%) as an orange–red powder. ¹H NMR (CDCl₃) δ 8.62 (d, $J = 7.8$ Hz, 2 H), 7.91 (t, $J = 6.1$ Hz, 2 H), 7.37–7.32 (m, 10 H), 5.25

(s, 4 H), 4.83–4.80 (dt, $J = 7.6, 3.8$ Hz, 2 H), 4.33–4.29 (m, 2 H), 4.11 (dd, $J = 11.5, 4.1$ Hz, 2 H), 4.04–3.99 (m, 4 H), 3.89 (dd, $J = 8.6, 4.7$ Hz, 2 H), 3.77–3.72 (dt, $J = 14.0, 6.7$ Hz, 2 H), 3.44–3.39 (dt, $J = 13.7, 5.6$ Hz, 2 H), 3.17 (br, 2 H), 1.48 (s, 6 H), 1.35 (s, 6 H). ¹³C NMR (CDCl₃) δ 169.9, 165.8, 146.1, 135.2, 128.7, 128.5, 128.2, 125.9, 109.6, 75.4, 67.5, 67.1, 63.4, 54.9, 42.9, 27.2, 25.4. RP-LC/MS (ESI) m/z 781.4 (M + H)⁺ ($t_R = 4.80$ min, 5–95% B/6 min). HRMS (ESI) m/z calcd for C₃₈H₄₈N₆O₁₂Na (M + Na)⁺ 803.3222, found 803.3223; calcd for C₃₈H₄₈N₆O₁₂ (M + H)⁺ 781.3403, found 781.3412.

N²,N⁵-Bis[(R)-1,3-dihydroxy-1-oxopropan-2-yl]-3,6-bis{[(S)-2,3-dihydroxypropylamino]pyrazine-2,5-dicarboxamide (2j). To a solution of bis-acetonide 8e (0.075 g, 0.096 mmol) in THF (3 mL) was added 1.0 N HCl solution (0.5 mL) and stirred for 4 h at rt under argon atmosphere. Most of the THF was removed, and the aq reaction mixture was neutralized with 1.0 N NaOH solution and evaporated to dryness. RP-LC/MS analysis of at this stage indicated partial hydrolysis of benzyl ester groups even though the aliquot prior to neutralization with NaOH showed the presence of single peak with molecular ion at m/z 701.3 (M + H)⁺ corresponding to the desired product. To complete the ester hydrolysis, the above residue was dissolved in THF–H₂O (2 mL; 1:1, v/v) and treated with 1.0 N NaOH (1 mL) for 1 h at rt. The reaction mixture was neutralized with 1.0 N HCl, evaporated to dryness, and the crude product (0.200 g) was subjected to purification by gravity chromatography over C18 silica gel (gradient elution with water to 20% MeOH in water) to afford 2j (0.037 g, 74%) as a red powder. ¹H NMR (DMSO-*d*₆) δ 8.89 (d, $J = 5.7$ Hz, 2 H, exchangeable with D₂O), 7.90 (t, $J = 5.7$ Hz, 2 H, exchangeable with D₂O), 5.47 (br s, 2 H, exchangeable with D₂O), 4.89 (d, $J = 5.1$ Hz, 2 H, exchangeable with D₂O), 4.75 (t, $J = 5.7$ Hz, 2 H, exchangeable with D₂O), 3.84–3.80 (dt, $J = 7.4, 5.5$ Hz, 2 H), 3.77–3.72 (m, 4 H), 3.52–3.23 (m, 10 H). ¹³C NMR (DMSO-*d*₆) δ 172.67, 165.2, 145.9, 126.5, 70.3, 64.5, 62.7, 55.4, 44.6. RP-HPLC (280 nm) 98.2% ($t_R = 12.31$ min, 5–50% B/20 min). RP-LC/MS (ESI) m/z 521.3 (M + H)⁺ ($t_R = 3.03$ min, 5–95% B/6 min). HRMS (ESI) m/z calcd for C₁₈H₂₆N₆O₁₂ (M – H)[–] 519.1692, found 519.1680; calcd for C₁₈H₂₆N₆O₁₂ (M – 2H)^{2–} 259.0810, found 259.0802.

Measurement of Photophysical Properties and Protein Binding. In general, each test compound was dissolved in PBS buffer to form a 2 mM stock solution. The UV absorbance properties were determined on a 100 μ M solution in PBS using a UV-3101PC UV–vis–NIR scanning spectrophotometer system from Shimadzu. The fluorescence properties (λ_{ex} , λ_{em} , and intensity maximum at λ_{em}) were determined on a 10 μ M solution in PBS using a Fluorolog-3 spectrofluorometer system from Jobin Yvon Horiba. The percent plasma protein binding was determined on a 20 μ M compound solution in rat plasma incubated at 37 °C for 1 h. The separation of free from bound was made using an Amicon Centrifree YM-30 device (Regenerated Cellulose 30000 MWCO) and a Z400K refrigerated universal centrifuge from Hermle. The concentration of protein-free was determined via HPLC analysis using a set of external calibration standards and fluorescence detection.

Injected Dose Recovery in Urine Studies. Recovery of the injected dose in urine studies were conducted in either conscious or anesthetized Sprague–Dawley rats. The test compound (1 mL, 2 mM in PBS) was administered by tail vein injection into conscious, restrained rats, with subsequent collection of urine at the time points of 2, 4, and 6 h post injection. The metabolic cages were washed with water to maximize the recovery of urine discharged at each time point. Alternatively, rats were anesthetized with 100 mg/kg Inactin intraperitoneally, a trachea tube was inserted to maintain adequate respiration, and 1 mL of test compound was injected into the lateral tail vein. Rats were placed on 37 °C heating pad during the entire experiment. At 6 h post injection, the abdomen was opened, and the urine was removed from the bladder using a 21 gauge needle and a 3 cc syringe. Quantitation of each compound

in urine was performed via HPLC analysis using a set of external calibration standards and fluorescence detection. The percent recovery of compound in urine at each time point was calculated based on the balance of mass.

Invasive Pharmacokinetic Studies. Male Sprague–Dawley rats (330–380 g) were anesthetized by Inactin (IP). Rats were surgically instrumented with a trachea tube (PE-190) to facilitate breathing and femoral artery and vein catheters (PE-50 filled with 20 units/mL heparinized saline) for blood sampling and drug administration, respectively. After administration of 1 mL of a 2 mM solution of agent, approximately 200 μ L of blood was sampled and placed into a heparinized tube (Microtainer Brand Tube w/Lithium Heparin, BD 365971) at 0, 1, 6, 12, 18, 30, 45, 60, 90, 120 min. The concentration of compound in each centrifuged plasma sample was determined via HPLC analysis using a set of external calibration standards and fluorescence detection. The resulting pharmacokinetic parameters of the compound were analyzed using WinNonLin pharmacokinetic modeling software (Pharsight, Mountainview, CA).

Probenecid Inhibition Studies. Two groups of 4–6 rats male Sprague–Dawley rats were treated in the same manner as described above in the invasive pharmacokinetic studies. The treatment group received 70 mg/kg probenecid 10 min prior to injection of the test compound; this administration was flushed with 0.2 mL NaCl. The control group was treated with the vehicle (PBS).

Fluorescence Detection Studies. A Maestro in vivo imaging system from CRi was employed. Two mice were anesthetized and had their abdomens shaved and remaining hair removed with depilatory. A home-made catheter (30 gauge needle and PE 10 tubing) was put into the lateral tail vein for administration of the test materials. The green filter set of the Maestro instrument was used. A DyCE collection was set up with a 600 s duration at 1 frame every 3 s with a 2.5 ms exposure. A second time series was set up to acquire 1 frame every 60 s for a 3600 s duration. The collection was started approximately 10 s before the compounds were injected.

AUTHOR INFORMATION

Corresponding Author

*Phone: 314-654-3800. Fax: 314-654-8900. E-mail: Raghavan. Rajagopalan@covidien.com.

Present Addresses

[†]Department of Pharmaceutical Sciences, Southern Illinois University, 220 University Park Drive, Edwardsville, Illinois, 62026, United States.

ACKNOWLEDGMENT

We thank Dr. Carlos Rabito of the Harvard University Medical School and Massachusetts General Hospital, Professor Thomas Dowling of the University of Maryland, and Drs. Sevag Demirjian and Joseph Nally of the Cleveland Clinic Foundation for their insightful discussions and advice. We thank our Covidien Pharmaceutical colleagues Christopher R. DeFusco, Jolette Wojdyla, Tim Marzan, Micah Wilcox, Tasha Shoenstein, Rana Kumar, and Bich Vu for their experimental and analytical support. We thank our Covidien Pharmaceutical colleague Brian Donley for his help with the statistical analysis of the probenecid inhibition study. We thank Dr. Bryan Q. Spring of Wellman Center of Photomedicine for his help in imaging processing.

ABBREVIATIONS USED

GFR, glomerular filtration rate; %ID, percent injected dose; PPB, plasma protein binding; PBS, phosphate buffered saline; DTPA, diethylenetriaminepentaacetate

REFERENCES

- (1) Ekanoyan, G.; Levin, N. W. In *Clinical Practice Guidelines for Chronic Kidney Disease: Evaluation, Classification, and Stratification (K/DOQI)*; National Kidney Foundation: Washington, D.C., 2002; pp 1–22.
- (2) Rule, A. D.; Larson, T. S.; Bergstralh, E. J.; Slezak, J. M.; Jacobsen, S. J.; Cosio, F. G. Using serum creatinine to estimate glomerular filtration rate: accuracy in good health and in chronic kidney disease. *Ann. Intern. Med.* **2004**, *141*, 929–937.
- (3) Stevens, L. A.; Levey, A. S. Clinical implications of estimating equations for glomerular filtration rate. *Ann. Intern. Med.* **2004**, *141*, 959–961.
- (4) Bellomo, R.; Ronco, C.; Kellum, J. A.; Mehta, R. L.; Palevsky, P. Acute Dialysis Quality Initiative workgroup. Acute renal failure—definition, outcome measures, animal models, fluid therapy, and information technology needs: the Second International Consensus Conference of the Acute Dialysis Quality Initiative (ADQI) Group. *Crit. Care* **2004**, *8*, R204–R212.
- (5) Diskin, C. J. Creatinine and GFR: an imperfect marriage of convenience. *Nephrol., Dial., Transplant.* **2006**, *21*, 3338–3339.
- (6) Carrie, B. J.; Goldbetz, H. V.; Michaels, A. S.; Myers, B. D. Creatinine: an inadequate filtration marker in glomerular disease. *Am. J. Med.* **1980**, *69*, 177–182.
- (7) Stevens, L. A.; Levey, A. S. Measured GFR as a confirmatory test for estimated GFR. *J. Am. Soc. Nephrol.* **2009**, *20*, 2305–2313.
- (8) Sturgeon, C.; Sam, A. D.; Law, W. R. Rapid determination of glomerular filtration rate by single-bolus inulin: a comparison of estimation analyses. *J. Appl. Physiol.* **1998**, *84*, 2154–2162.
- (9) Wilson, D. M.; Bergert, J. M.; Larson, T. H.; Leidtke, R. R. GFR determined by nonradiolabeled iohalamate using capillary electrophoresis. *Am. J. Kidney Dis.* **1997**, *39*, 646–652.
- (10) Guesry, P.; Kaufman, L.; Orloff, S.; Nelson, J. A.; Swann, S.; Holliday, M. Measurement of glomerular filtration rate by fluorescent excitation of non-radioactive meglumine iohalamate. *Clin. Nephrol.* **1975**, *3*, 134–138.
- (11) Lewis, N.; Kerr, R.; Van Buren, C. Comparative evaluation of urographic contrast media, inulin, and ^{99m}Tc-DTPA clearance methods for determination of glomerular filtration rate in clinical transplantation. *Transplantation* **1989**, *48*, 790–796.
- (12) Rabito, C. A.; Chen, Y.; Schomacker, K. T.; Modell, M. D. Optical, real-time monitoring of the glomerular filtration rate. *Appl. Opt.* **2005**, *44*, S956–S965.
- (13) Chinen, L.; Galen, K. P.; Kuan, K. T.; Dyszlewski, M. E.; Ozaki, H.; Sawai, H.; Pandurangi, R. S.; Jacobs, F. G.; Dorshow, R. B.; Rajagopalan, R. Fluorescence-enhanced europium complexes for the assessment of renal function. *J. Med. Chem.* **2008**, *51*, 957–962.
- (14) Yu, W.; Sandoval, R. M.; Molitoris, B. A. Rapid determination of renal filtration using an optical ratiometric imaging approach. *Am. J. Physiol. Renal Physiol.* **2007**, *292*, F1873–F1880.
- (15) Schock-Kusch, D.; Sadick, M.; Henninger, N.; Kraenzlin, B.; Claus, G.; Kloetzer, H.-M.; Weiss, C.; Pill, J.; Gretz, N. Transcutaneous measurement of glomerular filtration rate using FITC-sinistrin in rats. *Nephrol., Dial., Transplant.* **2009**, *24*, 2997–3001.
- (16) Roch-Ramel, F.; Besseghir, K.; Murer, H. Renal excretion and tubular transport of organic anions and cations. In *Handbook of Physiology, Section 8, Neurological Physiology*, Vol. II; Windhager, E. E., Ed.; Oxford University Press: New York, 1992; pp 2189–2262.
- (17) Roch-Ramel, F.; De Broe, M. E. Renal handling of drugs and xenobiotics. In *Clinical Nephrotoxins*, 2nd ed.; De Broe, M. E., Porter, G. A., Bennet, W. M., Verpooten, G. A., Eds.; Kluwer Academic Publishers: Dordrecht, Netherlands, 2003; pp 21–46.
- (18) Preliminary results of the work were presented in part at the SPIE, Biophotonics West annual conference, San Jose, CA, 2008. Dorshow, R. B.; Asmelash, B.; Chinen, L. K.; Debreczeny, M. P.; Fitch, R. M.; Freskos, J. N.; Galen, K. P.; Gaston, K. R.; Marzan, T. A.; Poredddy, A. R.; Rajagopalan, R.; Shieh, J.-J.; Neumann, W. L. New molecular probes for the continuous monitoring of renal function. In *Molecular Probes for Biomedical Applications II*, Vol. 6867; Achilefu, S., Barnhop, D. J., Raghavachari, R., Eds.; SPIE Press: Bellingham, WA, 2008.

- (19) Fritzberg, A. R.; Kasina, S.; Eshima, D.; Johnson, D. L. Synthesis and biological evaluation of technetium-99m-MAG₃ as a hippuran replacement. *J. Nucl. Med.* **1986**, *27*, 111–116.
- (20) Shirai, K.; Yanagisawa, A.; Takahashi, H.; Fukunishi, K.; Matsuoka, M. Synthesis and fluorescent properties of 2,5-diamino-3,6-dicyanopyrazine dyes. *Dyes Pigm.* **1998**, *39*, 49–68.
- (21) Kim, J. H.; Shin, S. R.; Matsuoka, M.; Fukunishi, K. Self-assembling of aminopyrazine fluorescent dyes and their solid state spectra. *Dyes Pigm.* **1998**, *39*, 341–357.
- (22) Poreddy, A. R.; Asmelash, B.; Neumann, W. L.; Dorshow, R. B. A highly efficient method for the N-alkylation of aminopyrazines: Synthesis of hydrophilic red fluorescent dyes. *Synthesis* **2010**, 2383–2392.
- (23) Prueksaritanont, T.; Lui, C. Y.; Lee, M. G.; Chiou, W. L. Renal and non-renal clearances of iothalamate. *Biopharm. Drug Dispos.* **1986**, *7*, 347–355.
- (24) Bormans, G.; Cleynhens, B.; Adriaens, P.; de Roo, M.; Verbruggen, A. Synthesis and labeling characteristics of ^{99m}Tc-mercaptoacetyltripeptides. *J. Labelled Compound. Radiopharm.* **1993**, *33*, 1065–1078.
- (25) Bubeck, B.; Brandau, W.; Weber, E.; Kaelbe, T.; Parekh, N.; Georgi, P. Pharmacokinetics of technetium-99m-MAG₃ in humans. *J. Nucl. Med.* **1990**, *31*, 1285–1293.
- (26) Two-sample *t*-test. Weiss, N. A. *Introductory Statistics*, 8th ed.; Addison-Wesley: Reading, MA, 2007.
- (27) Dorshow, R. B.; Bugaj, J. E.; Burleigh, B. D.; Duncan, J. R.; Johnson, M. A.; Jones, W. B. Noninvasive fluorescence detection of hepatic and renal function. *J. Biomed. Opt.* **1998**, *3*, 340–345.