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Immucillins in custom catalytic-site cavities

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ABSTRACT

Neighboring-group participation in the reaction catalyzed by purine nucleoside phosphorylase involves a compression mode between the 5′- and 4′-ribosyl oxygens, facilitated by His257. The His257Gly mutant opens a space in the catalytic site. Hydrophobic 5′-substituted Immucillins are transition-state analogue inhibitors of this mutant enzyme. Dissociation constants as low as 2 pM are achieved, with K_m/K_d as high as 400,000,000.

Purine nucleoside phosphorylase (PNP)[1] catalyzes the phosphorolytic cleavage of inosine and guanosine as well as their 2′-deoxy analogues to (deoxy)ribose 1-phosphate and hypoxanthine or guanine (Fig. 1).

Inhibition of human PNP activity causes accumulation of deoxyguanosine, which in turn leads to downstream inhibition of cell division and apoptosis specifically in T-lymphocytes.[2–4] Thus, PNP has been identified as a target for the treatment of T-cell lymphoma, rheumatoid arthritis, psoriasis, multiple sclerosis, and other T-cell mediated disorders.[5]

The human PNP-catalyzed reaction has been shown to proceed through a dissociative transition state characterized by a ribooxacarbenium ion with a cationic C-1′, which is separated from the nucleobase by >3 Å.[6] These features were incorporated into a family of transition-state analogue inhibitors called Immucillins (Fig. 2), whose members typically possess low nanomolar to picomolar affinity for human PNP. The potent inhibitors exhibit slow-onset behavior, where a time-dependent conformational change converts the initial enzyme–inhibitor complex (E·I), with dissociation constant K_i, to an even more stable complex (E^*·I), with dissociation constant K_i^*. The first-generation inhibitor, Immucillin-H (ImmH, **1**),[7] has a K_i of 3.3 nM and a K_i^* of 58 pM.[8] Modification of ImmH produced second- and third-generation Immucillins represented by DADMe-ImmH (**4**)[9] and SerMe-ImmH (**9**),[10] respectively, with K_i^* values of 11 and 5.2 pM, respectively.

Mutation of His257 has shown that this residue serves an important role in transition-state formation by hydrogen-bonding with the 5′-OH, orienting it into an electron-rich 'oxygen stack' with O-4′ and O_P from the phosphate nucleophile (Fig. 3).[8] This interaction is reasoned to provide electron density to weaken the ribosidic bond and stabilize the developing cationic transition state. Though mutation adversely affected the steady-state properties of PNP, mutants were capable of binding ImmH and DADMe-ImmH with reasonably good affinity. Structural analysis revealed that one of the mutants, His257Gly, was capable of binding these inhibitors nearly identically to the native protein, despite side-chain removal (Fig. 4). We envisioned that the active-site cavity introduced in the His257Gly mutant could be exploited in the binding of bulkier Immucillin derivatives.

5′-Methylthio-ImmH (MeS-ImmH, **2**) and 5′-phenylthio-ImmH (PhS-ImmH, **3**)[11] were originally developed as specific inhibitors of PNP from *Plasmodium falciparum*, exhibiting 112- and 2-fold binding preference over human PNP, respectively.[12] With native human PNP, **2** and **3** bind with weaker affinity than **1**, showing no slow-onset behavior and yielding K_i values of 101 and 160 nM, respectively (Table 1). In comparison to the substrate inosine (K_m = 40 μM), modification of the 5′-hydroxyl of ImmH resulted in a drop in relative affinity (K_m/K_i) from 690,000 to 400 and 250, respectively. Mutation of His257 to glycine abolished the slow-onset character of ImmH (**1**), resulting in a K_i of 11.0 nM. Taken in light of the elevated K_m (750 μM) with this mutant, the relative affinity decreased 10-fold relative to the native enzyme [(mutant K_m/K_i)/(native K_m/K_i) = 0.099]. In the case of the bulkier derivatives **2** and **3**, however, not only was increased

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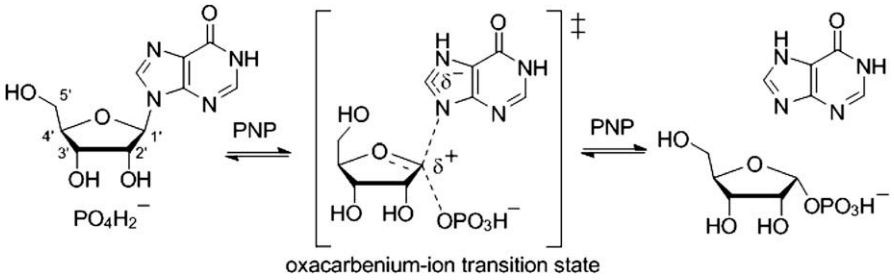


Figure 1. Phosphorolysis of inosine catalyzed by PNP.

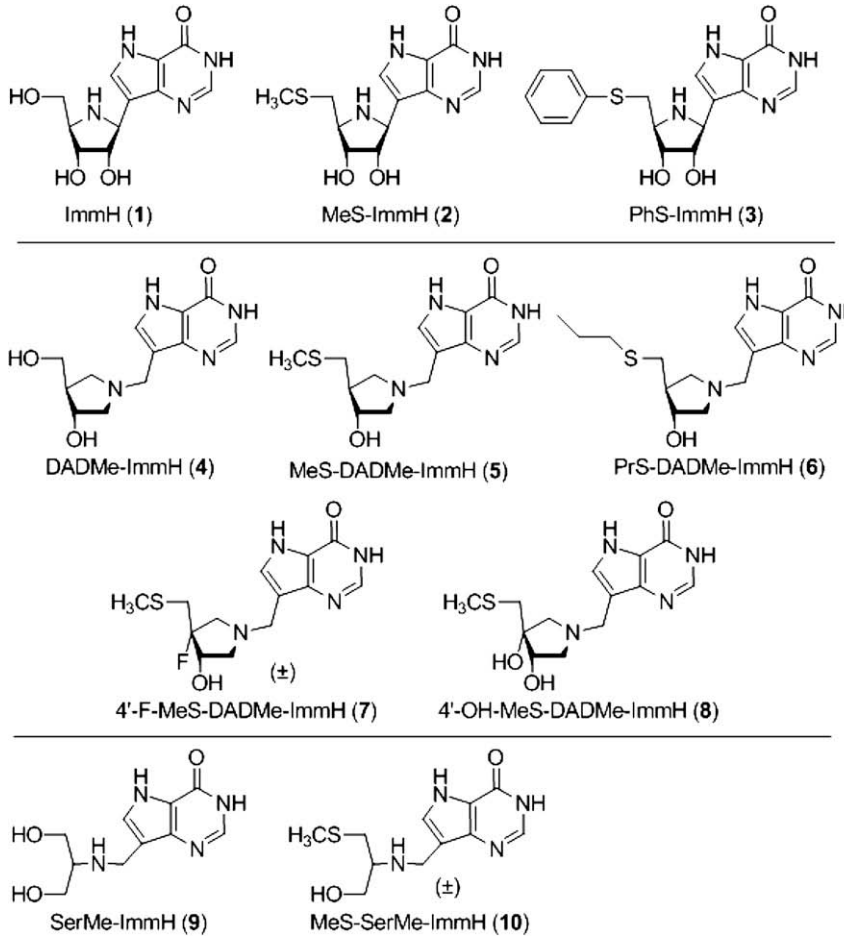


Figure 2. Three-generations of Immucillins, potent PNP transition-state analogue inhibitors. ImmH, DADMe-ImmH, and SerMe-ImmH are shown along with 5′-alkylthio and arylthio derivatives used in this study.

absolute affinity observed (K_i = 4.9 and 6.0 nM, respectively), but also the relative affinity increased to 150,000 and 120,000, respectively, up to 500-fold over native PNP.

The second-generation transition-state analogue DADMe-ImmH (**4**) was designed to mimic the human PNP transition state by increasing the leaving group distance through introduction of a methylene bridge between the pseudoribosidic bond and by moving the cationic ring nitrogen to the 1′-position, where significant positive-charge character is developed at the transition state.[13] With the native enzyme, DADMe-ImmH was found to be a more potent inhibitor than ImmH, giving a K_i^* of 10.7 pM.[8] Unlike the case with ImmH, the His257Gly mutant bound DADMe-ImmH in a slow-onset manner, with only slightly lowered relative affinity [(mutant K_m/K_i)/(native K_m/K_i) = 0.74]. 5′-Methylthio-DADMe-ImmH (MeS-DADMe-ImmH, **5**) and 5′-propylthio-DADMe-ImmH (PrS-DADMe-ImmH, **6**)[12] maintained strong potency with the native enzyme with K_i^* values of 19.6 pM (K_m/K_i = 2,000,000) and 9.8 pM (K_m/K_i = 4,100,000), respectively. These inhibitors were found to be strikingly effective with the His257Gly mutant, exhibiting slow-onset inhibition and yielding K_i^* values of only 2.8 and 1.9 pM, respectively. Not only are these values lower than the most potent inhibitor tested to date with native human PNP, but also the relative affinities (K_m/K_i) of 270,000,000 and 400,000,000 are the largest ever reported for any enzyme–inhibitor system. Inhibitors **5** and **6** are therefore bound with 130- and 97-fold preference, respectively, over the native protein.

Further derivatization of **5** to (±)-5′-deoxy-4′-fluoro-5′-methylthio-DADMe-ImmH (4′-F-MeS-DADMe-ImmH, **7**) was accomplished by functional group exchange from the fluorinated diol precursor **11**, followed by Mannich reaction of **13** with 9-deaza-

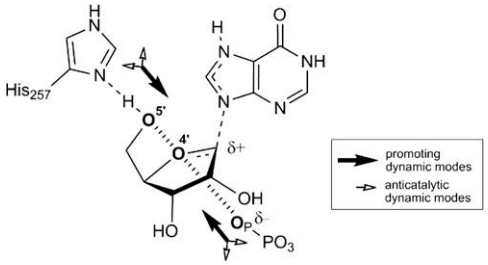


Figure 3. Proposed role of His257 in formation of the transition state, featuring dynamic compression of the O5′-O4′-O_P 'oxygen stack'. The 'oxygen stack' is represented by hashed bonds connecting bolded atoms, and arrows indicate promoting vibrational modes. Dashed bonds represent hydrogen bonds and partial bonds. Dynamic compressive motions (larger solid arrows) from the enzyme push O-5′ and the phosphate oxygen toward the ring oxygen, providing electron density to stabilize ribooxacarbenium-ion development and promote ribosidic bond fission.

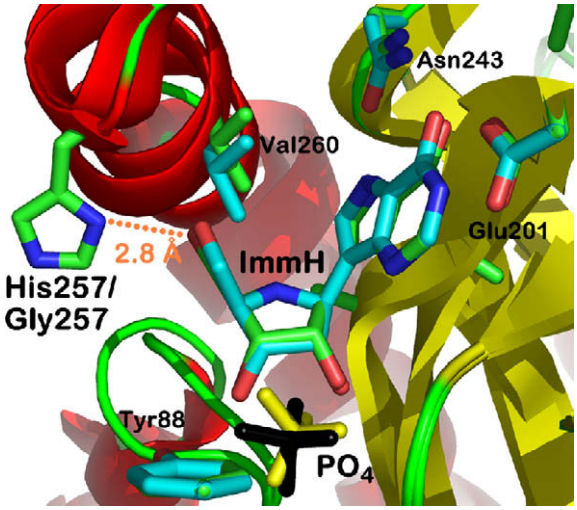
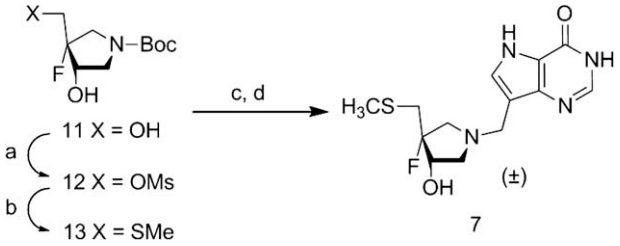


Figure 4. Overlay of the crystal structures of native human PNP and His257Gly complexed with ImmH and phosphate (PO_4). Side chains of selected active-site residues within 3.2 Å of ImmH have been included. Carbon atoms of these residues and of ImmH are green in the native enzyme and cyan in His257Gly. PO_4 is colored yellow and black in the native and mutant PNPs, respectively. The H-bond between His257 and the 5′-OH is indicated.

hypoxanthine and formaldehyde (Scheme 1).[14] Both the fluorinated derivative **7** and 5′-deoxy-4′-hydroxy-5′-methylthio-DADMe-ImmH (4′-OH-MeS-DADMe-ImmH, **8**)[12] resulted in the loss of slow-onset inhibition with native and mutant enzymes. These compounds bound nearly equally well to native PNP with relative affinities of 7500 and 5100, respectively. As in the case with **5** and **6**, when compounds **7** and **8** were tested with the glycine mutant, the dissociation constants dropped, resulting in enhanced relative affinities of 540,000 and 600,000, respectively, corresponding to 78- and 120-fold improvements over the native enzyme.



Scheme 1. Reagents and conditions: (a) Bu_2SnO, toluene, reflux, then MsCl, 81%; (b) NaSMe, DMF, 68%; (c) 6 N HCl; (d) 9-deazahypoxanthine, CH_2O, NaOAc, water–dioxane, 100 °C, 30%.

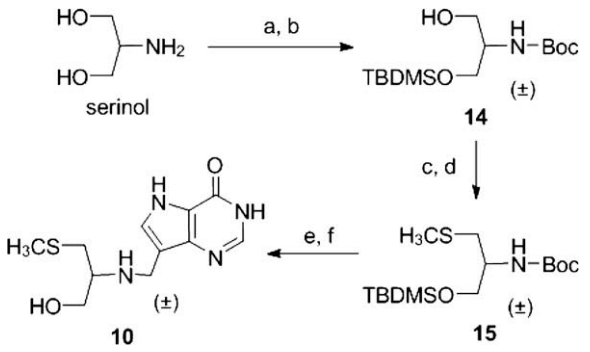
The third generation of PNP transition-state inhibitors consist of acyclic analogues of DADMe-ImmH. SerMe-ImmH (**9**), despite its lack of stereocenters, binds to human PNP with a K_i^* of 5.2 pM.[10] As was observed with ImmH, mutation of His257 resulted in the loss of slow-onset behavior, lowering the relative affinity from 7,700,000 to 260,000. The corresponding ratio of the relative affinities is 0.033, which is the lowest among the 10 inhibitors tested. SerMe-ImmH was also derivatized to the methylthio analogue **10** in a manner similar to that outlined for compound **7** above (Scheme 2).[15] As observed for compounds **7** and **8**, incubation of MeS-SerMe-ImmH (**10**) with native and mutant PNP lacked the slow-onset property of its underivatized analogue, yielding a lower K_m/K_i with the native protein (9300) but a larger value (680,000) with His257Gly. The discrimination for **10** by His257Gly is therefore 73-fold greater than that by native PNP.

This study has confirmed that human PNP tolerates substitution of the 5′-hydroxyl of the transition-state analogues ImmH, DADMe-ImmH, and SerMe-ImmH with alkylthio and arylthio groups; however, except for compounds **5** and **6**, the slow-onset nature of inhibition is lost, and inhibitor dissociation constants increase by two to three orders of magnitude. When the imidazole moiety of residue 257 is removed by mutation, the loss of an H-bond partner for the 5′-OH likely accounts for the observed decreases in binding affinity for the unmodified analogues **1**, **4**, and **9**.

Table 1
Dissociation constants of transition-state analogues with native human PNP and His257Gly[a]

Inhibitor	Native human PNP (inosine K_m = 40 μM)[b]			His257Gly (inosine K_m = 750 μM)[b]			Mutant K_m /K_i
	K_i (nM)	K_i^* (nM)[c]	K_m/K_i[d]	K_i (nM)	K_i^* (nM)[c]	K_m/K_i[d]	Native K_m/K_i
ImmH (**1**)[b]	3.3 ± 0.2	0.0579 ± 0.0015	690,000	11.0 ± 0.9	n/a	68,000	0.099
MeS-ImmH (**2**)	101 ± 4	n/a	400	4.9 ± 0.5	n/a	150,000	390
PhS-ImmH (**3**)	160 ± 15	n/a	250	6.0 ± 0.3	n/a	120,000	500
DADMe-ImmH (**4**)[b]	1.10 ± 0.12	0.0107 ± 0.0011	3,700,000	e	0.27 ± 0.02	2,800,000	0.74
MeS-DADMe-ImmH (**5**)	0.10 ± 0.02	0.0196 ± 0.0012	2,000,000	0.11 ± 0.02	0.0028 ± 0.0003	270,000,000	130
PrS-DADMe-ImmH (**6**)	0.117 ± 0.015	0.0098 ± 0.0007	4,100,000	0.066 ± 0.006	0.0019 ± 0.0002	400,000,000	97
4′-F-MeS-DADMe-ImmH (**7**)	5.8 ± 0.4	n/a	7500	1.40 ± 0.04	n/a	540,000	78
4′-OH-MeS-DADMe-ImmH (**8**)	7.9 ± 1.1	n/a	5100	1.26 ± 0.11	n/a	600,000	120
SerMe-ImmH (**9**)	0.11 ± 0.02	0.0052 ± 0.0004	7,700,000	2.93 ± 0.04	n/a	260,000	0.033
MeS-SerMe-ImmH (**10**)	4.3 ± 0.2	n/a	9300	1.10 ± 0.03	n/a	680,000	73

[a] All inhibition constants reported here were determined by the reported xanthine-oxidase-coupled assay (Ref. 12) using native or mutant enzyme from the same preparation.
[b] Values are from Ref. 8. All other values in this table are either newly determined or have been redetermined in this study to ensure reliable comparisons.
[c] K_i^* is the final, equilibrium dissociation constant for the slow-onset, tight-binding phase of inhibition. 'n/a' indicates that no slow-onset phase was observed.
[d] This ratio is K_m/K_i^* in cases where slow-onset inhibition occurs.
[e] The weak inhibition phase (K_i) was observed but too short to accurately quantitate, so only K_i^* is reported.



Scheme 2. Reagents and conditions: (a) Boc_2O, MeOH; (b) 1 equiv NaH, TBDMSCl, THF, 71% (2 steps); (c) MsCl, Et_3N, CH_2Cl_2; (d) NaSMe, DMF, 78% (2 steps); (e) HCl, MeOH, H_2O; (f) 9-deazahypoxanthine, CH_2O, NaOAc, H_2O, 80 °C, 28% (2 steps).

Nevertheless, the active-site cavity that is created accommodates bulkier functionalities which lack H-bonding opportunities. In all cases, the His257 mutant exhibited enhanced binding affinities relative to substrate for bulkier analogues over native PNP by factors ranging from 73 to 500. Unprecedented selective binding was observed with **5** and **6**, which associate up to 400 million times more tightly with His257Gly than inosine.

Acknowledgments

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References and notes

1. Abbreviations: PNP, purine nucleoside phosphorylase; ImmH, Immucillin-H; DADMe-ImmH, 4′-*d*eaza-1′-*a*za-2′-*d*eoxy-1′-(9-*m*ethylene)-ImmH; SerMe-ImmH, *ser*inol-*N*-(9-*m*ethylene)-ImmH.
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10. SerMe-ImmH (**9**) was prepared by reductive amination between serinol and 7-benzyloxymethyl-6-methoxy-9-carbaldehyde-9-deazahypoxanthine using sodium cyanoborohydride, followed by protecting-group removal with conc. HCl. Identity and purity were confirmed by NMR, ESI-MS, and elemental analysis. Full experimental details will be published elsewhere: Clinch, K.; Evans, G. B.; Furneaux, R. H.; Kelly, P. M.; Legentil, L.; Murkin, A. S.; Li, L.; Schramm, V. L.; Tyler, P. C.; Woolhouse, A. D., in preparation.
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13. Lewandowicz, A.; Tyler, P. C.; Evans, G. B.; Furneaux, R. H.; Schramm, V. L. *J. Biol. Chem.* **2003**, *278*, 31465.
14. Experimental procedure. *(±)-cis-1-tert-Butoxycarbonyl-4-fluoro-4-methylthiomethylpyrrolidin-3-ol (**13**):* Racemic pyrrolidinol **11** (Mason, J. M.; Murkin, A. S.; Li, L.; Schramm, V. L.; Gainsford, G. J.; Skelton, B. W. *J. Med. Chem.* in press) (0.52 g, 2.2 mmol) and dibutyltin oxide (0.66 g, 2.7 mmol) were heated to reflux in toluene under a Dean–Stark trap for 1 h. After cooling to rt, methanesulfonyl chloride (0.21 ml, 2.7 mmol) was added, and the resulting solution was stirred for 8 h. The solution was then applied to a column of silica gel. Elution with CH_2Cl_2/EtOAc (1:1) gave mesylate **12** (0.49 g, 1.78 mmol, 81%), which was taken up in DMF (7 ml) and stirred with sodium thiomethoxide (0.33 g, 4.7 mmol) for 2 h. The mixture was partitioned between water and diethyl ether, and the organic phase was concentrated under reduced pressure. Column chromatography (silica gel, 33% EtOAc in hexanes) gave the title compound **13** (0.28 g, 68% from **12**) as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$, TMS) δ 4.23–4.16 (br s, 1H), 3.89–3.41 (br m, 3H), 3.24 (m, 1H), 3.02–2.71 (br m, 3H), 2.23 (s, 3H), 1.46 (s, 9H). ^{13}C NMR (75.5 MHz, $CDCl_3$) (as mixture of invertomers, referenced to the center line of $CDCl_3$ at 77.0 ppm) δ154.3/154.1 (C), 101.7/100.9 (d, J = 180 Hz, C), 80.1 (C), 72.8/72.3 (d, J = 19 Hz, CH), 53.2/52.5, d, J = 27 Hz, CH_2), 50.2/49.3 (CH_2), 36.5 (d, J = 26 Hz, CH_2), 28.4 (CH_3), 17.8 (CH_3). ESI-MS $C_{11}H_{20}FNNaO_3S$ [M+Na]$^+$; Calcd 288.1046, found 288.1033.*(±)-cis-1-((9-Deazahypoxanthin-9-yl)methyl)-4-fluoro-4-(methylthiomethyl)pyrrolidin-3-ol (4′-F-MeS-DADMe-ImmH, 7):* A solution of **13** (43 mg, 0.16 mmol) in methanol (1 ml) and conc. HCl (0.5 ml) was evaporated to dryness under reduced pressure. The resulting deprotected pyrrolidinol was taken up in water (1.8 ml) and dioxane (0.2 ml). Sodium acetate (27 mg, 0.32 mmol), 9-deazahypoxanthine (33 mg, 0.24 mmol), and formaldehyde (37%, 0.026 ml, 0.32 mmol) were added and the solution was heated at 100 °C for 2.5 h. The solvents were removed under reduced pressure and the residue was chromatographed on a column of silica gel eluted with 20% methanolic ammonia (7 M) in CH_2Cl_2. Fractions containing compound **4** were concentrated under reduced pressure. Trituration of this residue with MeOH gave the title compound **4** (15 mg, 30%) as a white amorphous solid. ^{1}H NMR (DMSO-d_6, TMS) δ 11.96 (br s, 1H), 11.83 (br s, 1H), 7.80 (s, 1H), 7.30 (s, 1H), 5.03 (d, J = 6.8 Hz, 1H), 3.82 (m, 1H), 3.69 (m, 2H), 3.11–2.59 (m, 5H), 2.48 (m, 1H), 2.10 (s, 3H). ^{13}C NMR (DMSO-d_6, referenced to the solvent center line at 39.9 ppm) δ 154.0 (C), 143.8 (C), 141.7 (CH), 127.4 (CH), 118.0 (C), 113.0 (C), 101.7 (d, J = 186 Hz, CH), 73.1 (d, J = 17 Hz, CH_2), 60.4 (d, J = 24 Hz, CH_2), 57.9 (CH_2), 47.7 (CH_2), 38.9 (d, J = 26 Hz, CH_2), 17.1 (CH_3). ESI-MS $C_{13}H_{18}FN_4O_2S$ [M+H]$^+$; Calcd 313.1135, found 313.1136. Dissolution in methanolic HCl and concentration to dryness gave the hydrochloride salt.
15. Experimental procedure. *(±)-tert-Butyl 1-(tert-butyldimethylsilyloxy)-3-hydroxypropan-2-ylcarbamate (**14**):* Serinol (0.80 g, 8.78 mmol) and Boc anhydride (2.11 g, 9.66 mmol) were stirred together in MeOH (10 ml) at rt for 1 h, and the solvent was evaporated. The solid residue was dried over P_2O_5 under vacuum and then added in portions to a suspension of NaH (60%, 0.35 g, 8.78 mmol) in dry THF (10 ml) with cooling in an ice bath (McDougal, P. G.; Rico, J. G.; Oh, Y.-I.; Condon, B. D. *J. Org. Chem.*, **1986**, *51*, 3388). The mixture was stirred for 45 min, then TBDMSCl (1.32 g, 8.78 mmol) was added. After 2 h, H_2O (4 ml) was added and the mixture was diluted with Et_2O (60 ml), washed with brine, dried ($MgSO_4$), and the solvent was evaporated. Chromatography on silica gel (EtOAc/hexanes, 2:8) gave **14** as a colorless oil (1.89 g, 70.5%). ^{1}H NMR (300 MHz, $CDCl_3$, TMS) δ 5.15 (br s, partly exchanged to D_2O, 1H), 3.88–3.57 (m, 5H), 2.86 (br s, exchanged to D_2O, 1H), 1.45 (s, 9H), 0.90 (s, 9H), 0.08 (s, 6H). ^{13}C NMR (75.5 MHz, $CDCl_3$, referenced to the center line of $CDCl_3$ at 77.0 ppm) δ 156.0 (C), 79.6 (C), 64.1 (2 x CH_2), 52.6 (CH), 28.4 (CH_3), 25.8 (CH_3), 18.2 (C), −5.6 (CH_3). ESI-MS $C_{14}H_{31}NNaO_4Si$ [M+Na]$^+$ Calcd 328.1920, found 328.1913.*(±)-tert-Butyl 1-(tert-butyldimethylsilyloxy)-3-(methylthio)propan-2-ylcarbamate (**15**).* Methanesulfonyl chloride (0.57 ml, 7.31 mmol) was added to a stirred solution of **14** (1.86 g, 6.09 mmol) and Et_3N (1.28 ml, 9.13 mmol) in CH_2Cl_2 (15 ml) cooled in an ice bath. The mixture was warmed and stirred at rt for 30 min, then diluted with CH_2Cl_2 (60 ml) and washed with sat. aq $NaHCO_3$ (3× 20 ml), dried ($MgSO_4$), and the solvent was evaporated to give the crude mesylate. It was dissolved in DMF (10 ml), sodium thiomethoxide (0.85 g, 12.18 mmol) added, and the mixture was stirred at rt for 3 h. Et_2O (50 ml) was added and the mixture was washed with H_2O (4× 10 ml), and brine (10 ml), dried ($MgSO_4$), and the solvent was evaporated. Chromatography on silica gel (EtOAc/hexanes, 5:95) gave **15** as a colorless oil (1.59 g, 78%). ^{1}H NMR (300 MHz, $CDCl_3$, TMS) δ 4.89 (bd, partly exchanged to D_2O, J = 7.0 Hz, 1H), 3.86 (dd, J = 9.9, 2.9 Hz, 1H), 3.75 (br s, 1H), 3.64 (dd, J = 9.9, 4.1 Hz, 1H), 2.65 (d, J = 7.0 Hz, 2H), 2.14 (s, 3H), 1.45 (s, 9H), 0.90 (s, 9H), 0.06 (s, 6H). ^{13}C NMR (75.5 MHz, $CDCl_3$, referenced to the center line of $CDCl_3$ at 77.0 ppm) δ 155.3 (C), 79.4 (C), 62.7 (CH_2), 50.8 (CH), 35.2 (CH_2), 28.4 (CH_3), 25.9 (CH_3), 18.3 (C), 15.9 (CH_3), −5.5 (CH_3). ESI-MS $C_{15}H_{33}NNaO_3SSi$ [M+Na]$^+$ Calcd 358.1848, found 358.1846. *(±)-1′-Methylthio-SerMe-ImmH (**10**):* Compound **15** (0.40 g, 1.19 mmol) was dissolved in a 1:1 mixture of MeOH/37% aq HCl (4 ml) and left at rt for 1.5 h. The solvent was evaporated and the residue was dissolved in H_2O (2 ml) and NaOAc (0.11 g, 1.31 mmol), aq formaldehyde solution (37%, 0.32 ml, 3.99 mmol) and 9-deazahypoxanthine (0.16 g, 1.19 mmol) were added. The mixture was heated and stirred at 80 °C for 16 h, then concentrated onto silica gel. Chromatography (CH_2Cl_2/7 M NH_3–MeOH, 9:1 → 85:15) gave **10** as a colorless solid, which was converted to the HCl salt by evaporation from HCl (5% aq). Recrystallization (H_2O–EtOH) gave the HCl salt of **10** as a colorless, hygroscopic solid (0.103 g, 28.4%). Mp 224–225 °C. ^{1}H NMR (300 MHz, D_2O, referenced to internal acetone at 2.23 ppm) δ 8.74 (s, 1H), 7.86 (s, 1H), 4.61 (d, J = 14.2 Hz, 1H), 4.55 (d, J = 14.2 Hz, 1H), 4.07 (dd, J = 12.8, 3.6 Hz, 1H), 3.96 (dd, J = 12.8, 4.8 Hz, 1H), 3.58 (m, 1 H), 2.98–2.85 (m, 2H), 2.07 (s, 3H). ^{13}C NMR (75.5 MHz, D_2O, referenced to internal CH_3CN at 1.47 ppm) δ 153.7 (C), 144.9 (CH), 135.5 (C), 132.9 (CH), 118.7 (C), 104.4 (C), 58.9 (CH_2), 58.0 (CH), 39.2 (CH_2), 31.8 (CH_2), 15.2 (CH_3). ESI-MS $C_{11}H_{17}N_4O_2S$ [M+H]$^+$ Calcd 269.1072, found 269.1069.