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Synthesis and Biological Activity of Peptidyl Aldehyde Urokinase Inhibitors

Susan Y. Tamura[†], Michael I. Weinhouse, Christopher A. Roberts, Erick A. Goldman[‡], Kevin Masukawa, Susanne M. Anderson, Cheryl R. Cohen[§], Annette E. Bradbury, Vernon T. Bernardino, Steven A. Dixon, Michael G. Ma, Thomas G. Nolan* and Terence K. Brunck[†]

Corvas International, Inc., Department of Medicinal Chemistry, 3030 Science Park Road, San Diego, CA 92121, USA

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Abstract—Solid- and solution-phase synthesis of peptidomimetic inhibitors of urokinase-type plasminogen activator based on the sequence dSerAlaArg-al are described. The biological activities of these unique inhibitors are reported herein. Carbonate prodrugs were prepared and tested as potential drug delivery systems. © 2000 Published by Elsevier Science Ltd.

Urokinase-type plasminogen activator (u-PA) is one of the two major endogenous plasminogen activators that catalyze the conversion of the zymogen plasminogen to the fibrinolytic protease plasmin.[1] The primary role of u-PA is to generate plasmin in events involving the degradation of the extracellular matrix.[2] Localization of u-PA on the cell surface is achieved by binding to urokinase plasminogen activator receptor (u-PAR),[3] which is attached to the cell membrane via its glycosyl phosphatidyl inositol (GPI) anchor. Recent advances in the elucidation of the function of the u-PA/u-PAR system have led to an increased understanding of the role played by this enzyme in angiogenesis,[4] cell invasion,[5] and cancer metastasis.[6] Considerable efforts are being focused on the development of selective direct and mechanism-based synthetic u-PA inhibitors.[7] Inhibitors of u-PA are potential therapeutic targets for cancer, arthritis, and pathological angiopathies.[8]

A variety of tetrapeptide P_1-argininals were prepared using a library approach. The lead structures **1a** and **1b** were discovered by screening individual compounds in this library based on in vitro activity against human u-PA (Fig. 1). The solid- and solution-phase syntheses of peptidomimetic inhibitors of u-PA based on the tripeptide sequence of **1a** and **1b**, D-SerAlaArg-al, is described herein. The targets possess an interesting range of topographical and physical properties including polarity and lipophilicity, which further elucidated the requirements for potential inhibitors in the active site of u-PA. Many of these peptidyl aldehydes possess significant levels of potency against urokinase and selectivity towards other serine proteases, including t-PA.

Solid-Phase Chemistry[9]

Two solid-phase methodologies were used in the preparation of 2–10 mg quantities of final target argininals: the argininal aminal linker methodology and the peptidyl aldehyde-HCAM resin methodology.

The argininal aminal linker methodology[10] was utilized in the preparation of the targets **2, 5–7** and **9**. The preparation of compound **2** is exemplified in Scheme 1. Iterative TBTU/HOBt mediated couplings with the appropriate *N*-α-Fmoc-amino acids and piperidine deblocking protocols produced P_3–P_2 intermediate **14**, which was capped with a sulfonamide P_4 group to generate the fully elaborated intermediate **15**. The production of **2** from **15** was achieved in the following manner: catalytic removal of the *N*g-alloc moiety and subsequent acidic hydrolysis to cleave the aldehyde from the resin while

*Corresponding author. Tel.: +1-858-455-9800; fax: +1-858-455-7895; e-mail: tom_nolan@corvas.com
[†]Present address: Bioreason, Inc., 150 Washington Ave., Suite 303, Santa Fe, NM 87501, USA.
[‡]Present address: COR Therapeutics Inc., 256 E. Grand Ave., San Francisco, CA 94080, USA.
[§]Present address: Allergan Pharmaceuticals, 2525 Dupont Drive, Irvine, CA 92612, USA.

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removing the *t*-butyl protecting group from the serine hydroxyl.

The peptidyl aldehyde-HCAM resin methodology[11] incorporates an aldehyde protected as a semicarbazone that is directly attached to aminomethylpolystyrene resin. The peptidyl-argininal synthesis via the HCAM resin, similar in practice to the above mentioned argininal aminal solid phase iterative method, was utilized in the preparation of **3**, **4**, **8**, and **10**–**11**; however, the final targets were released from the resin using a different cleavage protocol. Beginning with Fmoc-Arg(Boc)$_2$-HCAM resin **16**,[11] piperidine deblocking protocols and iterative TBTU/HOBt mediated couplings produced the resin-bound intermediates. Final hydrolysis with 90% aqueous TFA concomitantly removed the peptide protecting groups and cleaved the target aldehydes **10** and **11** from the resin. In the case of compound **4** (Scheme 2), the capped P_3 amino acid, 2-phenethyl SO_2-D-Ser-OH, was prepared in solution before being coupled in this iterative manner.[12] Overall, the differences in the argininal aminal linker and argininal HCAM methodologies were found in both the ease of guanidine deprotection and higher yields using the HCAM method.

Solution-Phase Chemistry[9]

The preparation of milligram to multigram quantities of argininal **1a** for in vivo evaluation was facilitated by the solution-phase aminal methodology previously described wherein a protected argininal synthon, nitroargininal ethyl cyclol,[13] was coupled to a peptide surrogate (Scheme 3). After D-serine *t*-butyl ether was capped with isobutylchloroformate, the resulting iBoc-D-serine *t*-butyl ether was coupled to alanine *t*-butyl ester to afford compound **17**. The *N*-capped dipeptide **17** was deprotected with TFA, then coupled to nitroargininal ethyl cyclol. The nitro group of the fully elaborated target **18** was hydrogenolyzed, and the aminal was hydrolyzed under mild conditions to reveal the aldehyde. RP HPLC purification yielded the final target.

In Vitro Pharmacology: Results and Discussion

Eleven peptidyl argininals were prepared and evaluated for their ability to inhibit various serine proteases (Table 1). Potent and selective inhibitors of u-PA were discovered. From this focused set of compounds, we were able to discover several determinants for the

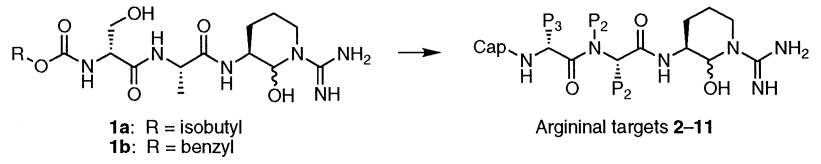
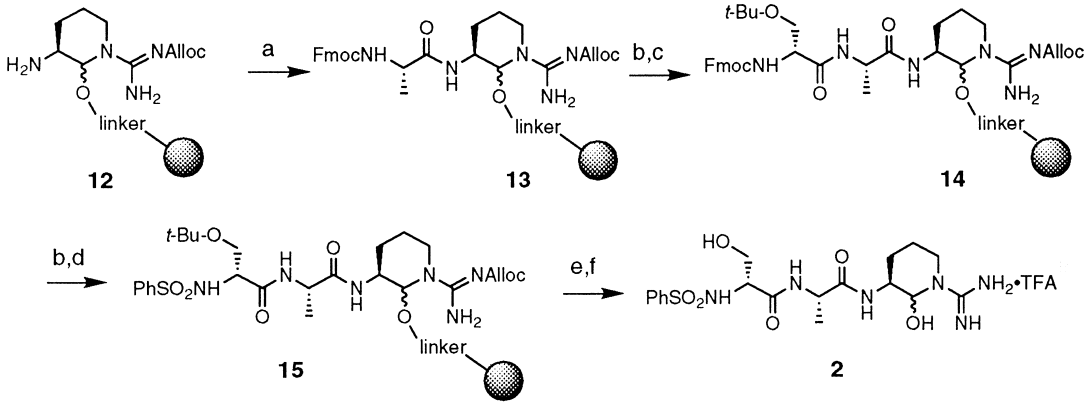
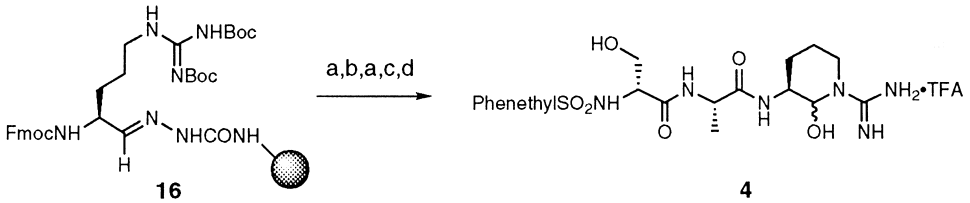


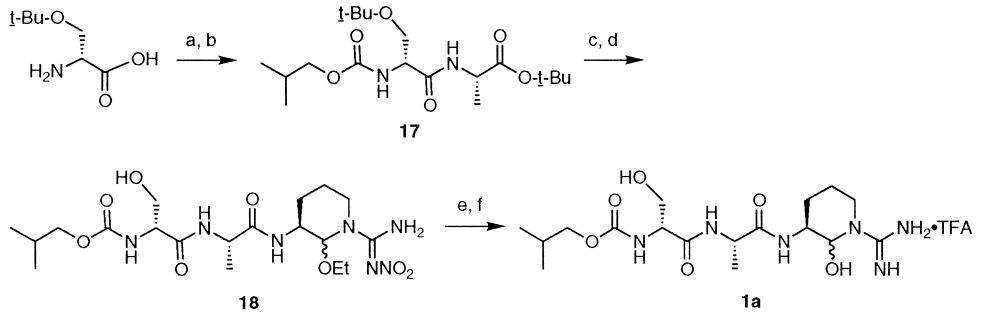
Figure 1. Design of new peptidyl original targets based on structures **1a** and **1b**.



Scheme 1. Reagents and conditions: (a) Fmoc-Ala-OH, TBTU, HOBt, DMF, DIEA; (b) 10% piperidine in DMF; (c) Fmoc-dSer(*t*-Bu)OH, TBTU, HOBt, DMF, DIEA; (d) benzenesulfonyl chloride, 2,4,6-collidine, CH_2Cl_2; (e) $Pd(PPh_3)_4$, morpholine, THF, DMSO, 0.5M HCl; (f) TFA: CH_2Cl_2:H_2O (6:3:1), 3–4 h; preparative RP HPLC.



Scheme 2. Reagents and conditions: (a) 10% piperidine in DMF; (b) Fmoc-Ala-OH, TBTU, HOBt, DMF, DIEA; (c) phenethylSO_2D-Ser(O-*t*-Bu)OH, TBTU, HOBt, DMF, DIEA; (d) TFA:H_2O (9:1); preparative RP HPLC.



Scheme 3. Reagents and conditions: (a) *i*-BuOCOCl, aq Na_2CO_3, 99%; (b) Ala *t*-butyl ester, EDC, HOBt, CH_3CN, DIEA, quant.; (c) TFA, CH_2Cl_2, quant.; (d) *N*g-nitroargininal ethyl cyclol, HCl salt, EDC, HOBt, CH_3CN, DIEA, 50%; (e) 50 psi H_2, 10% Pd/C, EtOH, HOAc, H_2O, 4 h; (f) 3.0 M HCl; preparative RP HPLC, 62% for two steps.

Table 1. In vitro IC_{50} values (nM) of peptidyl P_1 argininals against urokinase, plasmin, and *t*-PA[a]

Compound	Synthetic method[b]	P_4	P_3	P_2	Urokinase	Plasmin	*t*-PA
1a	C	*i*-Boc	d-Ser	Ala	23.1	1460	>2500
1b	A	Cbz	d-Ser	Ala	19.0	904	>2500
2	A	$PhSO_2$	d-Ser	Ala	10.3	1170	>2500
3[14]	B	$BnSO_2$	d-Ser	Ala	5.1	275	>2500
4	B	2-phenethylSO_2	d-Ser	Ala	3.1	367	>2500
5	A	Cbz	d-Thr	Ala	261	699	>2500
6	A	Cbz	d-alloThr	Ala	33.4	282	>2500
7	A	Cbz	d-Ser	Pip	101	—	>2500
8[14]	B	Cbz	d-Ser	Pro	12.4	125	>2500
9	A	Cbz	d-Ser	Aze	11.0	482	>2500
10	B	Cbz	d-Ser	d-Ala	>2500	>2500	>2500
11	B	Cbz	d-Me-d-Ser	Ala	>2500	>2500	>2500

[a]Concentration of compounds **1a**, **1b**, **2**–**12** necessary to inhibit human enzyme (urokinase, plasamin, and *t*-PA) cleavage of the chromogenic substrates described in ref 15 by 50%.
[b]Method A: aminal-linker solid-phase synthesis; Method B: semicarbazone solid-phase synthesis; Method C: solution-phase synthesis.

potency and selectivity of peptidyl argininal u-PA inhibitors. Changes in the P_4 position to sulfonamides from carbamates produced potent inhibitors, the best being the phenethylsulfonamide **4**. The stereochemistry of the hydroxyl group of P_3 is critical to binding when *threo*-nine replaces the serine: D-allo-Thr containing target **6** is 5-fold more potent than D-Thr derivative **5**. Surprisingly, replacement of D-Ser with an α-methyl-D-Ser in the P_3 position resulted in the abolished activity of target **11**. This may be due to differences in the ground state conformation of the quarternary C-α of **11**, including undesirable changes in the φ and ψ angles at the P_3 position. When cyclic amino acids were placed in the P_2 position, the following general trend in activities was observed: the smaller the ring size, the greater the potency of the target against u-PA. In this series, the P_2 analogues **8** and **9** were the most potent against u-PA; however, these compounds exhibited a decreased selectivity towards plasmin.

Prodrugs

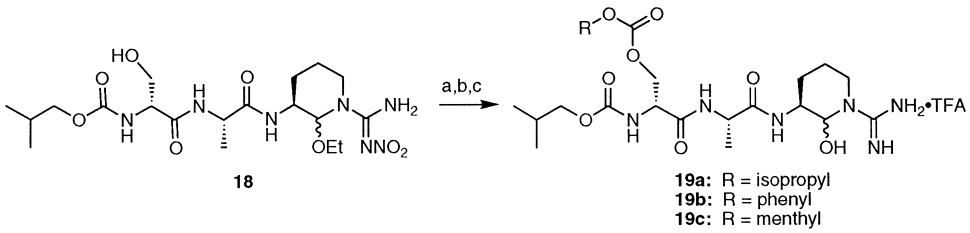
Although compound **1a** displayed a C_{max} of 22.9 ± 2.3 μg/mL and an overall AUC of 2333 ± 361 μg•min/mL in conscious dogs (20 mg/kg po, $n = 3$), its apparent terminal elimination half-life was only 51 ± 3.3 min.[16] Compound **1a** was administered sc to rats at a dose of 50 mg/kg ($n = 3$), and its plasma concentration was measured over 24 h. Pharmacokinetic analysis revealed an apparent terminal elimination half-life ($t_{1/2}$) of 300 ± 31 min, with a C_{max} of 4.7 ± 0.7 μg/mL (see Table 2). Further biological evaluation of this candidate was limited due to the short plasma half-life following oral or sc administration. Mechanisms to improve the pharmacokinetics, while retaining the high level of bioavailability of this general class of compounds, are under active study. The highly polar and hydrophilic nature of **1a** was proposed to have a detrimental effect on the plasma half-life of the inhibitors. Carbonate moieties possessing a broad range of physical properties including lipophilicity and polarizability may impart desirable pharmacokinetic properties upon prodrugs of this class of inhibitors. In addition, variations in the carbonate may affect its rate of hydrolysis or enzymatically catalyzed cleavage in vivo.

Prodrugs **19a**–**c** were prepared[17] (Scheme 4) in order to test the effect of carbonates on the in vivo plasma half-life of **1a**. The simple preparation of these compounds required the derivatization of the DSer hydroxyl of compound **18** with the appropriate chloroformate in pyridine. Hydrogenolysis of the nitro group followed by hydrolysis of the ethyl aminal produced the desired targets.

Prodrugs **19a**–**c** were easily converted to **1a** in plasma, where the half-life of the prodrugs was determined by

Table 2. Pharmacokinetic parameters of **1a** (mean ± sem): comparison of **1a** and **19a** dosed in rat ($n = 3$, 50 mg/kg sc)

Compound	Apparent terminal half-life of **1a** (h)	C_{max} (μg/mL)	T_{max} (h)	AUC (μg·min/mL)
1a	5.0 ± 0.52	4.7 ± 0.7	2.33 ± 0.88	2672 ± 351
Prodrug 19a	10.7 ± 2.4	2.3 ± 0.1	2.67 ± 0.67	2316 ± 49



18

19a: R = isopropyl
19b: R = phenyl
19c: R = menthyl

Scheme 4. Reagents and conditions: (a) ROCOCl, pyr, 45–62%; (e) 50 psi H_2, 10% Pd/C, EtOH, HOAc, H_2O, 4 h; (f) 3.0 M HCl; preparative RP HPLC, 15–33% for two steps.

analytical RP HPLC analysis. The half-life of **19a** ranged from a few minutes in rat and mouse plasma to 45–75 min in dog and human plasma. Facile cleavage of **19c** to **1a** was also observed in rat plasma. Surprisingly, it was found that prodrug moiety of **19b** was unstable at neutral pH in aqueous solution.

Prodrug **19a** was administered sc to rats at a dose of 50 mg/kg ($n = 3$), and plasma concentration of prodrug **19a** and **1a** were measured over 30 h.[16] As predicted, no **19a** was observed. The apparent terminal elimination half-life ($t_{1/2}$) of **1a** was 10.7 ± 2.4 h, with a C_{max} of 2.3 ± 0.1 μg/mL. The comparison of pharmacokinetic data of compound **1a** derived from dosing both **1a** (plasma concentration was measured over 24 h) and **19a** in rats is shown in Table 2. The major differences in the plasma concentration-time profile of **1a** following dosing with **19a** compared to **1a** are an extended duration and attenuated C_{max} with little effect on the overall exposure (relative bioavailability approximately 87%). The apparent terminal elimination half-life appeared to be longer after dosing with **19a**; however, the elimination phase was not linear and, since the mechanistic details of pro-drug conversion in vivo are not well understood, the apparent terminal elimination half-life should be interpreted with caution.

Conclusion

Solid-phase synthesis of peptidyl argininal targets has resulted in further delineation of the active site requirements of urokinase plasminogen activator and in the discovery of potent and selective inhibitors. Scaleup of **1a** and preparation of its prodrugs has allowed pharmacokinetic analysis of compound **1a** in rats. This information will be useful in dosing rats for in vivo efficacy studies. Further investigation in animal models of angiogenesis, tumor angiogenesis and tumor invasion is ongoing and will be reported in due course.

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12. (a) D-Ser methyl ester, HCl salt, was converted to 2-phenylethylSO$_2$-DSer(*O*-*t*-Bu)-OH in two steps: capping with phenethylsulfonyl chloride and collidine in acetonitrile, and hydrolysis of the methylester with 1.0 N LiOH in aqueous dioxane. (b) Compound **3** was prepared in an analogous manner.
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14. Compounds **3** and **8** were spectroscopically and chromatographically homogeneous ($\geqslant$90% RP HPLC).
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16. The determination of plasma levels in animals was accomplished using solid-phase extraction sample preparation with HPLC mass spectrometry to quantify compound **1a** and its prodrugs using methodologies that will be published elsewhere. The data were evaluated for pharmacokinetic parameters using WinNonlin noncompartmental analysis.
17. Compounds **19a**–**c** are isolated as an 85:15 mixture of L-Ala to D-Ala isomers.