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PAPER

Design and synthesis of amidine-type peptide bond isosteres: application of nitrile oxide derivatives as active ester equivalents in peptide and peptidomimetics synthesis†

Eriko Inokuchi,[a] Ai Yamada,[a] Kentaro Hozumi,[b] Kenji Tomita,[a] Shinya Oishi,[a] Hiroaki Ohno,[a] Motoyoshi Nomizu[b] and Nobutaka Fujii*[a]

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Amidine-type peptide bond isosteres were designed based on the substitution of the peptide bond carbonyl (C=O) group with an imino (C=NH) group. The positively-charged property of the isosteric part resembles a reduced amide-type peptidomimetic. The peptidyl amidine units were synthesized by the reduction of a key amidoxime (*N*-hydroxyamidine) precursor, which was prepared from nitrile oxide components as an aminoacyl or peptidyl equivalent. This nitrile oxide-mediated C–N bond formation was also used for peptide macrocyclization, in which the amidoxime group was converted to peptide bonds under mild acidic conditions. Syntheses of the cyclic RGD peptide and a peptidomimetic using both approaches, and their inhibitory activity against integrin-mediated cell attachment, are presented.

Introduction

The backbone modification of amide bonds **1** in bioactive peptides is one of the most promising approaches for improving their resistance towards degradation by peptidases.[1] A number of peptide bond isosteres that reproduce their electrostatic properties and secondary structure conformations have been reported.[2] Reduced amide bonds (–CH_2–NH–) **2** with a positively-charged secondary amine provide a flexible and hydrogen bond-donating substructure (Fig. 1). The success of this substructure is exemplified by several enzyme inhibitors of HIV-1 protease[3] and neuronal nitric oxide synthase.[4] Alkene dipeptide isosteres (–CR=CH–, R = H, F or Me) **3**[2,5] also represent steady-state peptide bond mimetics. This motif has been employed for the preparation of functional probes to identify indispensable peptide bonds. During the course of our medicinal chemistry studies using these isosteres, it has been demonstrated that a heavy atom corresponding to the carbonyl oxygen in peptide bonds favorably modulates local and global peptide conformations.[6]

The uncharged form of amidines **4** resemble the peptide bond structure **1**, in which both imino and amino functional groups share an sp^2 carbon. Under physiological conditions, amidines are protonated and the positive charge of the conjugated acid is delocalized over two nitrogens. Characteristic substructure **4′** can be viewed as a modified motif of the peptide bond and/or reduced amide structure. However, there are few reports on amidine-type peptide bond isosteres **4**,[7,8] while acyclic amidines[9] and cyclic amidines[10] have been utilized as equivalents of the basic guanidino group for several bioactive molecules.

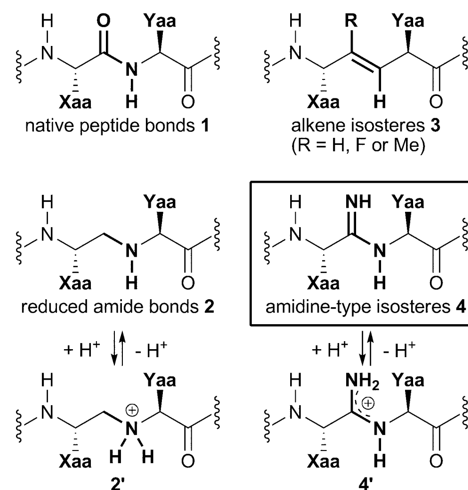

Fig. 1 Structures of the peptide bond and mimetics.

Whereas amidines have been synthesized directly by the Pinner reaction[7,8b] or by the coupling of imidyl chlorides with amines, these reactions are not applicable to peptidyl amidine synthesis because of the harsh reaction conditions or the arduous substrate preparation. We have postulated that amidoximes (*N*-hydroxyamidines) **8** represent an appropriate key precursor for peptidyl amidine synthesis, which are obtained by the coupling of nitrile oxides **6** with nucleophilic amines **7** (Scheme 1).[11] Reduction[10] or hydrolysis under mild acidic conditions[12] of the key

[a]*Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, 606-8501, Japan. E-mail: nfujii@pharm.kyoto-u.ac.jp; Fax: +81 75-753-4570; Tel: +81 75-753-4551*

[b]*School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo, 192-0392, Japan*

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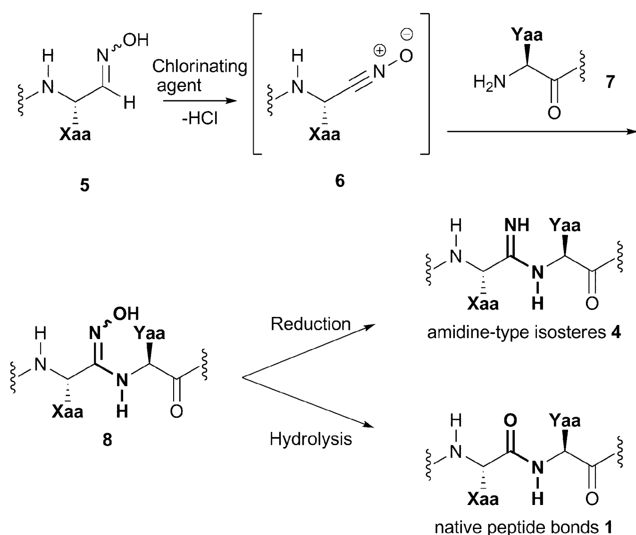


Scheme 1 Synthetic scheme for amidine-type peptide bond isosteres **4** and native peptide bonds **1** using nitrile oxides **6** as reactive acyl equivalents.

amidoximes **8** would provide the target peptidyl amidine **4** or the parent peptide bond **1**, respectively. It was also expected that highly reactive nitrile oxides **6** derived from peptide aldoximes **5** could be exploited as active ester equivalents for fragment condensation to prepare various protected peptides and peptidomimetics. Herein, we describe a novel approach for the synthesis of peptides and amidine-type peptidomimetics *via* peptide amidoximes.

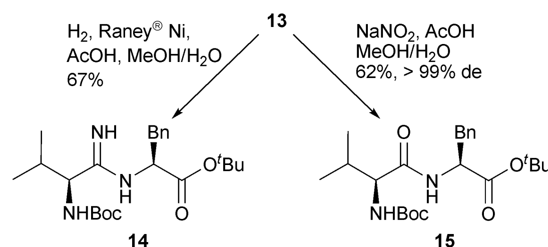
Results and discussion

Preparation of amino acid-derived nitrile oxides and their application to the synthesis of peptide bond and amidine-type peptidomimetics

Nitrile oxides are useful reactive species that can be formed from aldoximes by treatment with a chlorinating agent and a weak base.[11] There have been a number of reports of 1,3-dipolar cycloadditions of nitrile oxides with olefins to produce isoxazoline derivatives,[13] whereas examples utilizing nitrile oxides as active ester equivalents are limited. We expected that α-aminoaldoximes and peptide aldoximes would serve as useful precursors of reactive nitrile oxide components for peptide and peptidomimetic synthesis.

Initially, we optimized the coupling conditions of α-aminoaldoxime **9**[14] and α-amino ester **12**. This consisted of a two-step process including chlorination of aldoxime **9** and the subsequent nucleophilic attack of amino ester **12** onto nitrile oxide **11**, derived from **10**, under basic conditions (Table 1). The major isomer of *N*-Boc-valine aldoxime, **9a**, reacted with an NaOCl solution[11] followed by work-up and treatment with amino ester **12** to give the desired amidoxime (*N*-hydroxyamidine) product, **13**, in 77% yield (Table 1, entry 1), while minor isomer **9b** produced a complex mixture of unidentified products with the same reagent (Table 1, entry 2). This was presumably due to the concomitant formation of unstable nitrile oxide **11** under the basic conditions of the first chlorination step. Treatment of both aldoxime isomers **9a** and **9b** with *N*-chlorosuccinimide (NCS) in DMF without base provided the same product, **13**, in satisfactory yields (Table 1, entries 3 and 4). Of note, the chlorination of **9a** with NCS in $CHCl_3$ did not work, resulting in the recovery of the starting material. As such, a facile protocol to prepare amidoximes from the both isomers of amino acid-derived aldoximes was established.

Conversion processes from amidoxime **13** were next investigated. The hydrogenation of **13** with RANEY® Ni[10] cleaved the N–O bond to afford the expected amidine-type isosteric unit, **14**, in 67% yield (Scheme 2). Alternatively, the hydrolysis of **13** under mild acidic conditions containing $NaNO_2$ gave the parent dipeptide unit, **15**, in 62% yield.[12] No epimerization of the amidoximes occurred during the coupling process, which was verified by comparing **15** with two authentic diastereomers prepared by the standard protocol for peptide synthesis.



Scheme 2 Conversion of *N*-hydroxyamidine **13** to amidine **14** and peptide bond **15**.

Table 1 Optimization of the aldoxime–amino acid coupling conditions

step a: chlorinating agent, DMF, rt. — **9a** (major) / **9b** (minor) → [**10** → **11**] — step b: H-Phe-O^{t}Bu **12** (1 equiv.), Et_3N, solvent, rt. → **13**

Entry	Substrate[a]	Step a	Step b	Yield (%)
1	**9a**	NaOCl[b] (3.0 equiv.), Et_3N (3.0 equiv.)	Et_3N (6.0 equiv.)/CH_2Cl_2	77
2	**9b**	NaOCl[b] (3.0 equiv.), Et_3N (3.0 equiv.)	Et_3N (6.0 equiv.)/CH_2Cl_2	Decomp.
3	**9a**	NCS (1.4 equiv.)	Et_3N (4.0 equiv.)/Et_2O	71[c]
4	**9b**	NCS (1.4 equiv.)	Et_3N (4.0 equiv.)/Et_2O	81

[a] Substrates **9** were prepared from Boc-valinal according to literature procedures.[14] [b] 30% aqueous solution. [c] When $CHCl_3$ was used as the reaction solvent in step a, starting material **9a** was recovered.

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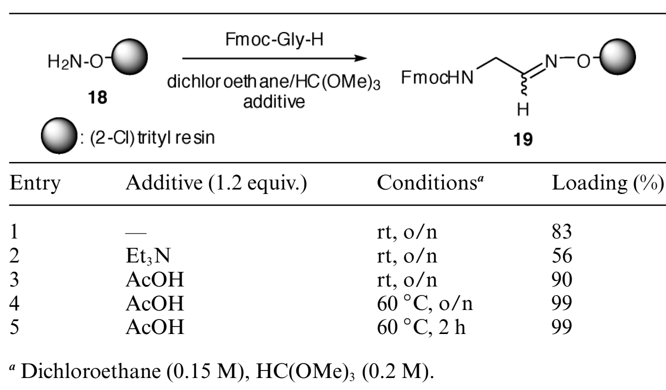
Table 2 Preparation of aldoxime resin **19**



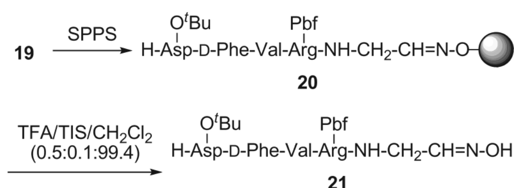
Entry	Additive (1.2 equiv.)	Conditions[a]	Loading (%)
1	—	rt, o/n	83
2	Et_3N	rt, o/n	56
3	AcOH	rt, o/n	90
4	AcOH	60 °C, o/n	99
5	AcOH	60 °C, 2 h	99

[a] Dichloroethane (0.15 M), $HC(OMe)_3$ (0.2 M).

Solid-phase synthesis of peptide aldoximes and the application of nitrile oxide-mediated coupling to cyclic peptide synthesis

The nitrile oxide-mediated synthesis of peptides and peptidomimetics was applied by a solid-phase approach. We chose cyclic RGD peptide **16**, *cyclo*(–Arg–Gly–Asp–D-Phe–Val–),[15] which is a highly potent integrin $\alpha_v\beta_3$ antagonist that includes two reactive side-chains (Arg and Asp), and mimetic **17** *cyclo*(–Arg–Gly–ψ[C(=NH)–NH]–Asp–D-Phe–Val–) as target peptides. We planned to synthesize RGD peptide **16** and peptidomimetic **17** by a nitrile oxide-mediated cyclization, followed by hydrolysis and hydrogenolysis, respectively. For application to solid-phase synthesis, the preparation of aldoxime resin **19** was investigated. The direct attachment of Fmoc-protected aminoaldoximes such as Fmoc–NH–CH_2–CH=NH–OH onto the (2-Cl)trityl chloride resin failed to afford the expected resin under any conditions. In contrast, resin **18** was prepared from the (2-Cl)trityl chloride resin and Fmoc-protected hydroxyamine (89% loading), followed by piperidine treatment. The reaction of an Fmoc-protected α-aminoaldehyde with aminooxy (2-Cl)trityl resin **18** gave the desired aldoxime resin, **19** (83% loading; Table 2, entry 1). An acidic additive improved the reactivity, and the reaction proceeded smoothly at 60 °C within 2 h to give aldoxime resin **19** in 99% yield (Table 2, entry 5).[16]

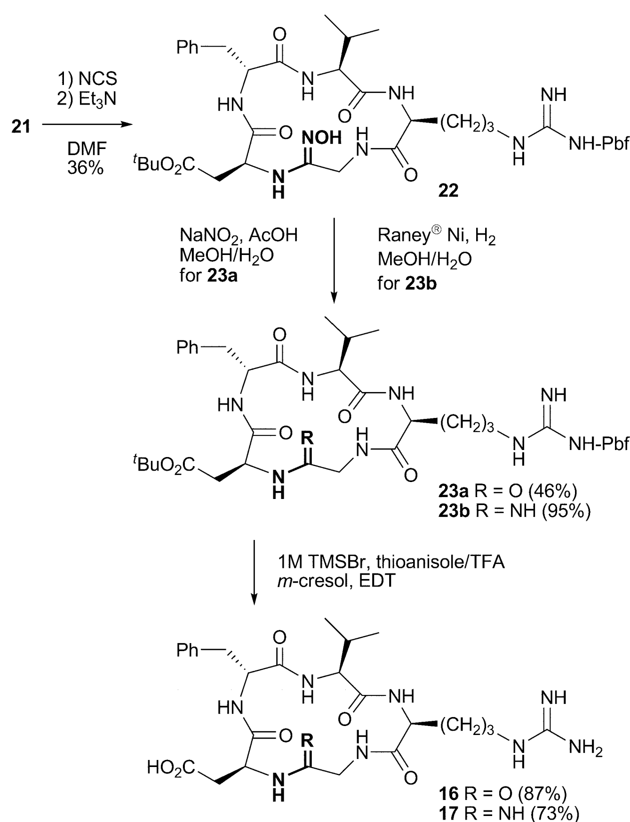
Peptide elongation was performed by the standard Fmoc-based solid-phase synthesis approach using *N*,*N′*-diisopropylcarbodiimide (DIC)/HOBt in DMF to give peptide aldoxime resin **20** (Scheme 3). During the solid-phase process, the oxime–ether linker was inert, even when treated with 20% piperidine in DMF for Fmoc removal. For peptide cleavage from the solid support, the standard conditions [30% 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP) in CH_2Cl_2, rt, 2 h[17]] were ineffective for aldoxime resin **20**, indicating that the oxime–ether linkage is less acid-labile compared with peptide acids and peptide alcohols. The treatment of resin **20** in TFA/triisopropylsilane



Scheme 3 Preparation of peptide aldoxime **21**.

(TIS)/CH_2Cl_2 (0.5/0.1/99.4) provided linear peptide aldoxime **21** in a quantitative yield.

Cyclization of acyclic peptide aldoxime **21** by treatment with NCS, followed by Et_3N, gave amidoxime-containing peptide **22** in a moderate yield (36%, Scheme 4). The yield of the aldoxime-mediated cyclization was comparable with approaches using the azide method or DPPA-mediated cyclizations (11–52% cyclization yields for the RGD peptide **16** and the derivatives).[15a] Subsequently, amidoxime **22** was converted smoothly to amide **23a** and amidine **23b** in 46 and 95% yields, respectively, by $NaNO_2$-mediated acidic hydrolysis and RANEY® Ni-mediated hydrogenation. The protecting groups for Arg and Asp were cleaved off using a cocktail of 1 M TMSBr–thioanisole/TFA in the presence of *m*-cresol and 1,2-ethanedithiol (EDT) in a short time, providing the desired parent RGD peptide **16** and peptidomimetic **17** in 87 and 73% yields, respectively. It is of note that no hydrolyzed product **16** was observed during the deprotection treatment of **23b** and subsequent HPLC purification process.



Scheme 4 Synthesis of cyclic RGD peptide **16** and amidine-type isosteric congener **17**.

Biological activity of the cyclic RGD Peptide with an amidine-type isosteric unit for the Gly-Asp dipeptide

The resulting cyclic RGD peptidomimetic **17** was evaluated for its inhibitory effect of integrin-mediated cell attachment (Fig. 2). Peptide **17**, with an amidine moiety, showed moderate inhibitory activity (IC_{50} = 4.77 μM) compared with original peptide **16** (peptide **16**, IC_{50} = 0.157 μM). The X-ray crystal structure of the $\alpha_v\beta_3$ integrin-cyclic RGD peptide complex indicated that the

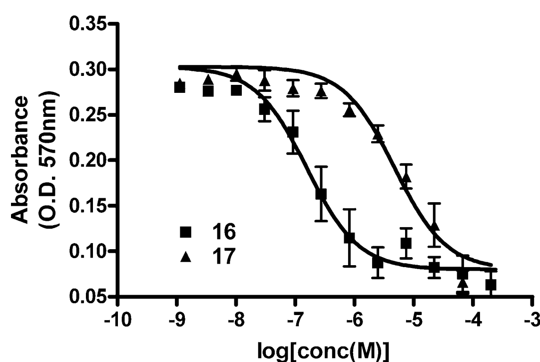


Fig. 2 The inhibitory effect of cyclic RGD peptides on HDF attachment to vitronectin. HDFs were allowed to attach to human vitronectin in the presence of various concentrations of cyclic RGD peptides. Peptides were added to the cell suspension and the cells were plated. After a 30 min incubation period, the attached cells were stained with crystal violet and dissolved in a 1% SDS solution. The absorbance at 570 nm was measured. Triplicate experiments gave similar results.

uncharged amide NH of Gly-Asp is located proximal to the integrin residue Arg216, which is likely to be involved in the interaction.[18] These results suggest that substitution of the Gly-Asp peptide bond with a positively-charged amidine unit partially eliminated the highly potent binding affinity towards the $\alpha_v\beta_3$ integrin.

Conclusion

In conclusion, we have established a novel approach to synthesize acyclic amidine and amide units *via* a key amidoxime (*N*-hydroxyamidine) precursor, which was prepared from a nitrile oxide component as an active ester equivalent. This method was used for the Fmoc-based solid-phase synthesis of peptides and peptidomimetics containing an amidine-type isostere. The peptide aldoxime represented a functional precursor for a protected cyclic peptide and peptidomimetic, suggesting that the nitrile oxide-mediated coupling reaction could serve as an alternative method for peptide macrocyclizations. Further studies on the scope and limitations of this approach, as well as applications for structure–activity relationship studies of bioactive peptides, are currently in progress.

Experimental section

Synthesis

***tert*-Butyl [(*S*)-1-(hydroxyiminomethyl)-2-methylpropyl]carbamate (9).** To a solution of Boc-Val-NMe(OMe) (5.00 g, 19.2 mmol) in Et_2O (60 cm^3) was added dropwise a solution of $LiAlH_4$ (1.02 g, 27.0 mmol) in Et_2O (20 cm^3) at −40 °C and the mixture was stirred for 40 min. The reaction was quenched at −40 °C by the addition of an Na_2SO_4 solution. The reaction mixture was washed with saturated aqueous $NaHCO_3$ and brine, and dried over Na_2SO_4. Concentration under reduced pressure gave the Boc-valinal. To a solution of $NH_2OH{\cdot}HCl$ (1.66 g, 23.9 mmol) and AcONa (1.96 g, 23.9 mmol) in EtOH (50 cm^3) was added the solution of the aldehyde in EtOH (15 cm^3). The reaction mixture was stirred at 80 °C for 15 min. The mixture was concentrated under reduced pressure. The residue was extracted with CH_2Cl_2, and the extract was washed with H_2O and dried over Na_2SO_4. Concentration under reduced pressure followed by flash chromatography over silica gel with n-hexane–EtOAc (3/1) gave the title compounds **9a** and **9b** (3.44 g, 82% yield, **9a**/**9b** = 58/42) both as white solids.

Compound **9a**: mp 35–36 °C; $[\alpha]_D^{26}$ +11.0 (*c* 0.58, $CHCl_3$); δ_H (500 MHz, DMSO, Me_4Si) 0.82 (6H, dd, J = 13.7 and 6.9 Hz), 1.37 (9H, s), 1.75 (1H, td, J = 13.7 and 6.9 Hz), 3.72–3.78 (1H, m), 6.96 (1H, d, J = 8.8 Hz), 7.14 (1H, d, J = 7.3 Hz) and 10.63 (1H, s); δ_C (125 MHz, DMSO-d_6, Me_4Si) 18.6, 18.8, 28.2 (3C), 30.9, 55.4, 77.7, 149.0 and 155.1. Anal. calc. for $C_{10}H_{20}N_2O_3$: C, 55.53; H, 9.32; N, 12.95. Found: C, 55.29; H, 9.17; N, 12.81%.

Compound **9b**: mp 114–115 °C; $[\alpha]_D^{26}$ +50.0 (*c* 0.18, $CHCl_3$); δ_H (500 MHz, DMSO-d_6, Me_4Si) 0.81 (6H, t, J = 7.2 Hz), 1.37 (9H, s), 1.76–1.85 (1H, m), 4.54 (1H, dd, J = 15.7 and 7.1 Hz), 6.51 (1H, d, J = 7.1 Hz), 6.95 (1H, d, J = 8.9 Hz) and 10.86 (1H, s); δ_C (125 MHz, DMSO, Me_4Si) 18.3, 18.7, 28.2 (3C), 30.7, 50.2, 77.7, 149.9 and 155.2. Anal. calc. for $C_{10}H_{20}N_2O_3$: C, 55.53; H, 9.32; N, 12.95. Found: C, 55.25; H, 9.32; N, 12.71%.

***tert*-Butyl (*S*)-2-{[(*S*)-2-*tert*-butoxycarbonylamino-*N*-hydroxy-3-methylbutanimidoyl]amino}-3-phenylpropionate (13).** To a solution of aldoxime **9b** (30.0 mg, 0.140 mmol) in DMF (0.6 cm^3) was added *N*-chlorosuccinimide (26.2 mg, 0.200 mmol) and the mixture stirred at room temperature for 4 h. The reaction mixture was extracted with EtOAc, the extract washed with a solution of H_2O/brine (1/1) and dried over Na_2SO_4. After concentration under reduced pressure, the residue was dissolved in Et_2O (5 cm^3). To the solution were added Et_3N (77 mm^3, 0.560 mmol) and H-Phe-O^{t}Bu **12** (30.0 mg, 0.140 mmol), and the mixture stirred at room temperature overnight. The reaction mixture was washed with brine and dried over Na_2SO_4. Concentration under reduced pressure followed by flash chromatography over silica gel with n-hexane–EtOAc (3/1) gave title compound **13** (50.0 mg, 81% yield, inseparable mixture of major/minor = 97/3) as a colorless oil: $[\alpha]_D^{26}$ −19.2 (*c* 0.73, $CHCl_3$); δ_H (500 MHz, DMSO-d_6, Me_4Si) 0.63 (3H, d, J = 6.6 Hz), 0.73 (3H, d, J = 6.6 Hz), 1.32 (9H, s), 1.37 (9H, s), 1.79 (1H, dt, J = 21.7 and 6.6 Hz), 2.84–2.94 (2H, m), 3.70 (1H, t, J = 9.0 Hz), 4.44–4.52 (1H, m), 5.38 (1H, d, J = 10.5 Hz), 6.66 (1H, d, J = 9.5 Hz), 7.19–7.29 (5H, m) and 10.86 (1H, s); δ_C (125 MHz, DMSO-d_6, Me_4Si) 18.3, 19.8, 27.5 (3C), 28.2 (3C), 29.8, 55.0, 56.1, 77.8, 80.6, 126.5, 128.0 (3C), 129.5 (2C), 137.0, 150.5, 155.3 and 171.4; HRMS (FAB) m/z calc. for $C_{23}H_{38}N_3O_5$ ($[M + H]^+$) 436.2811, found 436.2808.

***tert*-Butyl (*S*)-2-{[(*S*)-2-*tert*-butoxycarbonylamino-3-methylbutanimidoyl]amino}-3-phenylpropionate (14).** To a solution of amidoxime **13** (29.1 mg, 0.0670 mmol) in MeOH (1 cm^3) and AcOH (0.011 cm^3) was added RANEY® Ni (0.85 cm^3, slurry in H_2O) and the mixture stirred under an atmosphere of hydrogen at room temperature for 1 h. The mixture was filtered through Celite®. Concentration under reduced pressure followed by flash chromatography over silica gel with n-hexane–EtOAc (3/1) gave title compound **14** (18.9 mg, 67% yield) as a yellow oil: $[\alpha]_D^{26}$ +7.53 (*c* 0.46, $CHCl_3$); δ_H (500 MHz, DMSO-d_6, Me_4Si) 0.76 (6H, dd, J = 13.5 and 6.7 Hz), 1.28 (9H, s), 1.38 (9H, s), 1.80–1.88 (1H, m), 2.86 (1H, br s), 2.92 (1H, dd, J = 13.5 and 6.9 Hz), 3.77 (1H, br s), 4.26 (1H, br s), 4.99 (1H, d, J = 9.5 Hz), 6.16 (1H, br s), 6.96 (1H, d, J = 9.5 Hz) and 7.14–7.26 (5H, m); δ_C (125 MHz, DMSO-d_6, Me_4Si) 18.0 (2C), 19.3, 27.5 (3C), 28.2 (3C), 31.0, 37.8, 59.5, 77.8,

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78.9, 126.1, 127.9 (2C), 127.9, 129.2 (2C), 138.1, 155.2 and 171.2; HRMS (FAB) m/z calc. for $C_{23}H_{38}N_3O_4$ ([M + H]$^+$) 420.2862, found 420.2864.

***tert*-Butyl (*S*)-2-[(*S*)-2-*tert*-butoxycarbonylamino-3-methylbutyrylamino]-3-phenylpropionate (15).** To a solution of amidoxime **13** (35.3 mg, 0.0810 mmol) in MeOH (0.8 cm^3) and H_2O (0.8 cm^3) were added AcOH (0.00800 cm^3, 0.120 mmol) and $NaNO_2$ (8.30 mg, 0.120 mmol). The mixture was stirred at room temperature overnight. The mixture was concentrated under reduced pressure. The residue was extracted with CH_2Cl_2, and the extract was washed with H_2O and dried over Na_2SO_4. Concentration under reduced pressure followed by flash chromatography over silica gel with n-hexane–AcOEt (3/1) gave title compound **15** (21.0 mg, 62% yield) as a white solid: mp 115–116 °C; $[\alpha]_D^{24}$ +60.0 (c 0.87, $CHCl_3$); δ_H (500 MHz, DMSO-d_6, Me_4Si) 0.87 (3H, d, J = 5.6 Hz), 0.93 (3H, d, J = 6.8 Hz), 1.38 (9H, s), 1.45 (9H, s), 2.04–2.14 (1H, m), 3.04–3.11 (2H, m), 3.91 (1H, t, J = 6.8 Hz), 4.74 (1H, dd, J = 13.8 and 6.2 Hz), 5.16 (1H, d, J = 6.6 Hz), 6.30 (1H, d, J = 6.2 Hz) and 7.14–7.31 (5H, m); δ_C (125 MHz, DMSO-d_6, Me_4Si) 17.7, 19.2, 27.9 (3C), 28.3 (3C), 38.2, 52.2, 53.6, 55.1, 82.2, 82.3, 126.9, 127.0 (2C), 128.4 (2C), 129.5, 136.0, 170.3 and 171.0; HRMS (FAB) m/z calc. for $C_{23}H_{37}N_2O_5$ ([M + H]$^+$) 421.2702, found 421.2702.

H_2N–O-(2-Cl)Trt resin (18). 2-Chlorotrityl resin chloride (loading: 1.31 mmol g^{-1}, 76.3 mg) was reacted with Fmoc–NHOH (128 mg, 0.500 mmol) and pyridine (0.0810 cm^3, 1.00 mmol) in THF (0.8 cm^3) at 60 °C for 6 h. The solution was removed by decantation and the resulting resin washed with a solution of DMF/(^{i}Pr)$_2$NEt/MeOH (17/2/1). The Fmoc protecting group was removed by treating the resin with a DMF/piperidine solution (80/20, v/v). The loading was determined by measuring at 290 nm the UV absorption of the piperidine-treated sample: 0.900 mmol g^{-1}, 89%.

H–Asp(O^{t}Bu)–D-Phe–Val–Arg(Pbf)–Gly–aldoxime-(2-Cl)Trt resin (20). Solid-supported hydroxyamine **18** (loading: 0.900 mmol g^{-1}, 91.6 mg, 0.0820 mmol) was reacted with Fmoc-glycinal (0.500 mmol) in dichloroethane (0.7 cm^3), $HC(OMe)_3$ (0.5 cm^3) and AcOH (0.001 cm^3) at 60 °C for 2 h. The solution was removed by decantation and the resulting resin was washed with DMF to afford resin **19**. Peptide resin **20** was manually constructed using an Fmoc-based solid-phase synthesis on resin **19**. The Fmoc protecting group was removed by treating the resin with a DMF/piperidine solution (80/20, v/v). The Fmoc-protected amino acid (0.500 mmol, 6.1 equiv.) was successively condensed using 1,3-diisopropylcarbodiimide (0.0770 cm^3, 0.500 mmol, 6.1 equiv.) in the presence of N-hydroxybenzotriazole (77 mg, 0.500 mmol, 6.1 equiv.) to give resin **20**. The ^{t}Bu ester for Asp and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg were employed for side-chain protection.

H–Asp(O^{t}Bu)–D-Phe–Val–Arg(Pbf)–Gly–aldoxime (21). Resin **20** was treated with TFA/TIS/CH_2Cl_2 (20 cm^3, 0.5/0.1/99.4) at room temperature for 1.5 h. After removal of the resin by filtration, the filtrate was concentrated under reduced pressure to give crude peptide aldoxime **21** as a yellow oil (74.0 mg, quant. from resin **18**). The crude product was used without further purification.

***Cyclo*[–Arg(Pbf)–Gly–ψ[C(=NOH)NH]–Asp(O^{t}Bu)–D-Phe–Val–] (22).** To a solution of peptide aldoxime **21** (74.0 mg) in DMF (1 cm^3) was added N-chlorosuccinimide (14.7 mg, 0.100 mmol). The solution was stirred at room temperature overnight, and then DMF (40 cm^3) and Et_3N (0.4 cm^3) added. The mixture was stirred at room temperature overnight and then concentrated under reduced pressure. The residue was extracted with EtOAc and the extract washed with brine. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give a yellow oil, which was purified by column chromatography over silica gel with CH_2Cl_2–MeOH (95/5) to give **22** (26.9 mg, 36% yield, major/minor = 79/21) as a yellow solid: mp 168–169 °C; $[\alpha]_D^{25}$ −52.7 (c 0.28, $CHCl_3$); δ_H (500 MHz, DMSO-d_6, Me_4Si) 0.85 (major, 3H, d, J = 6.9 Hz), 0.68 (minor, 3H, t, J = 6.4 Hz), 0.73 (major, 3H, d, J = 6.7 Hz), 0.72–0.76 (minor, 3H, m), 1.25–1.50 (2H, m), 1.34 (minor, 9H, s), 1.37 (major, 9H, s), 1.36 (minor, 6H, s), 1.41 (major, 6H, s), 1.74–1.76 (2H, m), 2.00 (3H, s), 2.41 (3H, s), 2.47 (3H, s), 2.30–2.50 (2H, m), 2.59 (1H, dd, J = 15.9 and 5.9 Hz), 2.82–2.91 (2H, m), 2.96 (2H, s), 3.79 (major, 1H, t, J = 7.0 Hz), 3.82–3.88 (minor, 1H, m), 3.98 (major, 1H, dd, J = 14.7 and 7.7 Hz), 4.02–4.08 (minor, 1H, m), 4.10–4.14 (minor, 1H, m), 4.16–4.25 (major, 1H, m), 4.50 (major, 1H, dd, J = 14.6 and 8.4 Hz), 4.35–4.45 (minor, 1H, m), 4.54–4.65 (major, 1H, m), 4.60–4.75 (minor, 1H, m), 5.17 (minor, 1H, d, J = 9.6 Hz), 5.27 (major, 1H, d, J = 10.6 Hz), 6.37 (major, 1H, br s), 6.70 (minor, 1H, br s), 7.12–7.33 (5H, m), 7.42–7.53 (1H, m), 8.05–8.14 (2H, m), 8.32 (minor, 1H, d, J = 7.3 Hz), 8.45 (major, 1H, d, J = 5.9 Hz), 9.17 (minor, 1H, s) and 9.63 (major, 1H, s); δ_C (125 MHz, DMSO-d_6, Me_4Si) 12.1, 12.3, 17.3 (minor), 17.6 (major), 17.8 (major, 2C), 17.9 (minor, 2C), 18.9, 19.0 (major), 19.1 (minor), 21.1, 27.7 (3C), 27.7, 28.3 (major, 2C), 28.8 (minor, 2C), 36.3, 42.5 (2C), 52.0, 52.6, 55.0, 59.8 (minor), 60.3 (major), 62.8, 79.7 (minor), 80.3 (major), 86.3, 116.3, 124.3, 126.5, 128.1 (minor, 2C), 128.2 (major, 2C), 129.1 (major, 2C), 129.3 (minor, 2C), 131.4, 134.2, 137.0, 137.3, 148.9, 156.0, 157.5, 169.3 (major), 169.5 (minor), 170.7, 171.2, 172.2 and 172.4; HRMS (FAB) m/z calc. for $C_{43}H_{64}N_9O_{10}S$ ([M + H]$^+$) 898.4497, found 898.4502.

***Cyclo*[–Arg(Pbf)–Gly–Asp(O^{t}Bu)–D-Phe–Val–] (23a).** To a solution of amidoxime **22** (20.0 mg, 0.0220 mmol) in MeOH (0.5 cm^3) and H_2O (0.2 cm^3) were added AcOH (0.00500 cm^3) and $NaNO_2$ (4.60 mg, 0.0660 mmol). The mixture was stirred at room temperature overnight. The mixture was concentrated under reduced pressure. The residue was extracted with EtOAc, and the extract washed with H_2O and dried over $MgSO_4$. Concentration under reduced pressure followed by PTLC purification with CH_2Cl_2–MeOH (95/5) gave title compound **23a** (8.90 mg, 46% yield) as a white solid: mp 247–248 °C; $[\alpha]_D^{25}$ −32.3 (c 0.27, MeOH); δ_H (500 MHz, DMSO-d_6, Me_4Si) 0.70 (6H, dd, J = 20.7 and 6.7 Hz), 1.18–1.50 (2H, m), 1.34 (9H, s), 1.41 (6H, s), 1.65–1.72 (1H, m), 1.80–1.88 (1H, m), 2.01 (3H, s), 2.36 (1H, dd, J = 15.7 and 8.9 Hz), 2.41 (3H, s), 2.46 (3H, s), 2.80 (1H, dd, J = 13.7 and 6.6 Hz), 2.91–3.06 (2H, m), 2.96 (2H, s), 3.28 (2H, s), 3.82 (1H, t, J = 7.6 Hz), 4.00–4.10 (2H, m), 4.54–4.62 (2H, m), 6.35 (1H, br s), 6.70–6.80 (1H, m), 7.13–7.28 (5H, m), 7.42–7.50 (5H, m), 7.74 (2H, dd, J = 11.5 and 8.3 Hz), 7.95 (1H, d, J = 8.3 Hz), 8.06 (1H, d, J = 7.6 Hz) and 8.36 (1H, dd, J = 7.3 and 4.4 Hz); δ_C (125 MHz, DMSO-d_6, Me_4Si) 12.3, 17.6, 18.2, 18.9, 19.2, 25.8, 27.6 (3C), 28.3 (2C), 28.4, 29.7, 36.4, 37.1, 39.8, 42.5, 43.1, 48.9,

52.2, 53.9, 60.1, 80.0, 86.3, 116.3, 119.7, 124.3, 126.2, 128.1 (2C), 129.0 (2C), 130.3, 131.4, 137.3, 156.0, 157.4, 169.1, 169.4, 169.9, 170.8, 171.0 and 171.1; HRMS (FAB) *m/z* calc. for $C_{43}H_{63}N_8O_{10}S$ ([M + H]$^+$) 883.4388, found 883.4397.

***Cyclo*[–Arg(Pbf)–Gly–ψ[C(=NH)NH]-Asp(O^{t}Bu)–D-Phe–Val–] (23b).** To a solution of amidoxime **22** (30.0 mg, 0.0330 mmol) in MeOH (0.6 cm^3) and AcOH (0.006 cm^3) was added RANEY® Ni (0.440 cm^3, slurry in H_2O), and the mixture stirred under a H_2 atmosphere at room temperature for 2 h. The mixture was filtered through Celite®. Concentration under reduced pressure followed by flash chromatography over silica gel with CH_2Cl_2–MeOH (95/5) gave title compound **23b** (27.4 mg, 95% yield) as a colorless oil: $[\alpha]_D^{26}$ –53.3 (*c* 0.14, $CHCl_3$); δ_H (500 MHz, CD_3OD, Me_4Si) 0.74 (6H, dd, *J* = 14.7 and 6.9 Hz), 1.43 (9H, s), 1.45 (6H, s), 1.45–1.52 (1H, m), 1.55–1.60 (1H, m), 1.82–1.89 (1H, m), 1.95–2.00 (1H, m), 2.07 (3H, s), 2.56 (3H, s), 2.59 (1H, d, *J* = 6.6 Hz), 2.77 (1H, dd, *J* = 16.5 and 6.9 Hz), 2.94 (1H, dd, *J* = 13.3 and 6.7 Hz), 2.99 (2H, s), 3.05 (1H, dd, *J* = 13.2 and 9.0 Hz), 3.11–3.18 (1H, m), 3.53 (1H, d, *J* = 15.2 Hz), 3.87 (1H, d, *J* = 6.9 Hz), 4.28 (1H, d, *J* = 15.2 Hz), 4.34–4.37 (1H, m), 4.39–4.45 (1H, m), 4.68 (1H, dd, *J* = 9.0 and 6.9 Hz) and 7.15–7.29 (5H, m); δ_C (125 MHz, CD_3OD, Me_4Si) 12.5, 18.4, 18.7, 19.6, 19.7, 28.4, 28.4, 28.4 (3C), 29.6 (2C), 30.9, 37.9, 38.4, 44.0, 49.5, 49.7, 54.0, 56.4, 62.5, 82.6, 87.7, 118.5, 126.0, 127.9, 129.6 (2C), 130.4 (2C), 132.4, 133.5, 134.4, 138.0, 139.4, 158.1, 160.0, 172.0, 173.3, 173.6, 173.9, 174.2 and 174.3; HRMS (FAB) *m/z* calc. for $C_{43}H_{62}N_9O_9S$ ([M – H]$^-$) 880.4397, found 880.4395.

***Cyclo*[–Arg–Gly–ψ[C(=NH)NH]–Asp–D-Phe–Val–] (17).** Protected amidine **23b** (7.90 mg, 0.00900 mmol) was treated with 1 M TMSBr–thioanisole in TFA (10 cm^3) in the presence of *m*-cresol (0.1 cm^3) and 1,2-ethanedithiol (0.5 cm^3) at 4 °C for 15 min. The mixture was poured into ice-cold dry Et_2O (50 cm^3). The resulting powder was collected by centrifugation and washed three times with ice-cold dry Et_2O. The crude product was purified by preparative HPLC to afford expected peptide **17** as a white powder (5.30 mg, 0.00660 mmol, 73% yield): $[\alpha]_D^{25}$ –129.2 (*c* 0.17, MeOH); δ_H (500 MHz, DMSO-d_6, Me_4Si) 0.70 (3H, d, *J* = 6.6 Hz), 0.74 (3H, d, *J* = 6.6 Hz), 1.32–1.60 (3H, m), 1.73–1.84 (1H, m), 1.88–1.98 (1H, m), 2.59 (1H, dd, *J* = 17.0 and 5.7 Hz), 2.78 (1H, dd, *J* = 13.5 and 6.5 Hz), 2.84 (1H, dd, *J* = 17.2 and 8.2 Hz), 3.00 (1H, dd, *J* = 13.0 and 8.4 Hz), 3.04–3.13 (2H, m), 3.72–3.78 (2H, m), 3.90–3.98 (1H, m), 4.23 (1H, dd, *J* = 13.5 and 8.2 Hz), 4.43 (1H, t, *J* = 16.2 and 7.0 Hz), 4.53–4.60 (1H, m), 4.62–4.68 (1H, m), 6.80–7.40 (2H, br s), 7.16–7.28 (5H, m), 7.72 (1H, t, *J* = 5.7 Hz), 7.93 (1H, dd, *J* = 11.3 and 8.4 Hz), 8.12 (1H, d, *J* = 7.7 Hz), 8.28–8.32 (1H, m), 8.53 (1H, d, *J* = 7.7 Hz), 8.92–8.98 (1H, m), 9.10–9.20 (1H, m) and 9.64 (1H, s); δ_C (125 MHz, DMSO-d_6, Me_4Si) 17.9, 25.3, 28.2, 29.6, 34.2, 37.0, 37.1, 40.2, 51.7, 51.9, 54.2, 59.9, 126.4, 128.2 (2C), 129.1 (2C), 137.2, 156.8, 158.4, 164.8, 166.8, 170.7, 171.2, 171.3 and 171.7; HRMS (FAB) *m/z* calc. for $C_{26}H_{40}N_9O_6$ ([M + H]$^+$) 574.3102, found 574.3101.

***Cyclo*(–Arg–Gly–Asp–D-Phe–Val–) (16).** By an identical procedure to that described for the preparation of **17**, **23a** (8.00 mg, 0.00900 mmol) was converted into cyclic RGD peptide **16** (0.00790 mmol, 87% yield). All characterization data were in agreement with the data for the control peptide, which was synthesized using Fmoc-based solid-phase synthesis. $[\alpha]_D^{25}$ –21.6 (*c* 0.27, MeOH); δ_H (500 MHz, DMSO-d_6, Me_4Si) 0.68 (3H, d, *J* = 6.7 Hz), 0.75 (3H, d, *J* = 6.7 Hz), 1.32–1.45 (2H, m), 1.45–1.55 (1H, m), 1.69–1.80 (1H, m), 1.80–1.90 (1H, m), 2.38 (1H, dd, *J* = 16.4 and 5.5 Hz), 2.72 (1H, dd, *J* = 16.4 and 8.9 Hz), 2.81 (1H, dd, *J* = 13.5 and 6.1 Hz), 2.94 (1H, dd, *J* = 13.5 and 8.0 Hz), 3.05–3.14 (2H, m), 3.26 (1H, dd, *J* = 15.2 and 4.2 Hz), 3.82 (1H, t, *J* = 7.4 Hz), 4.04 (1H, dd, *J* = 15.2 and 7.7 Hz), 4.08–4.16 (1H, m), 4.55 (1H, dd, *J* = 14.2 and 7.2 Hz), 4.60–4.68 (1H, m), 6.58–7.11 (1H, br s), 7.15–7.25 (5H, m), 7.58 (1H, t, *J* = 5.7 Hz), 7.78 (1H, d, *J* = 7.4 Hz), 7.87 (1H, d, *J* = 8.0 Hz), 8.00 (1H, d, *J* = 7.4 Hz), 8.08 (1H, d, *J* = 8.6 Hz), 8.36 (1H, dd, *J* = 7.4 and 4.2 Hz) and 12.3 (1H, s); δ_C (125 MHz, DMSO-d_6, Me_4Si) 18.1, 19.1, 25.3, 28.2, 29.5, 34.8, 37.1, 40.2, 43.0, 48.8, 52.0, 53.9, 60.1, 126.1, 128.0 (2C), 129.0 (2C), 137.3, 156.6, 158.3, 169.4, 169.8, 170.6, 171.1 and 171.6; HRMS (FAB) *m/z* calc. for $C_{26}H_{39}N_8O_7$ ([M + H]$^+$) 575.2942, found 575.2952.

Evaluation of inhibitory activity against integrin-mediated cell attachment. Human dermal fibroblasts (HDFs; AGC Techno Glass, Chiba, Japan) were maintained in DMEM containing 10% FBS, 100 U cm^{-3} penicillin and 100 μg cm^{-3} streptomycin (Invitrogen, Carlsbad, CA, USA). Human plasma vitronectin (0.1 μg in 0.050 cm^3 well^{-1}; EMD Chemicals Inc., Gibbstown, NJ, USA) were added to 96-well plates (Nalge Nunc, Rochester, NY, USA) and incubated for 1 h at 37 °C. The plates were washed and blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) in DMEM. HDFs were incubated at room temperature for 15 min in various concentrations of peptides (0.001–200 μM in 1% DMSO). Then, 0.100 cm^3 HDFs (2×10^4 cells) in DMEM containing 0.1% BSA were added to each well and incubated at 37 °C for 30 min in 5% CO_2. The attached cells were stained with a 0.2% crystal violet aqueous solution in 20% MeOH (0.150 cm^3) for 15 min. After washing with Milli-Q water, the plates were dried overnight at room temperature and dissolved in 0.150 cm^3 of a 1% SDS solution. The absorbance at 570 nm was measured. Each sample was assayed in triplicate, and cells attached to the BSA were subtracted from all measurements. 1% DMSO did not have any effect on HDF attachment to vitronectin.

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