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PAPER

Expedient synthesis of pseudo-Pro-containing peptides: towards constrained peptidomimetics and foldamers†

Rossella De Marco, Alessandra Tolomelli, Marilena Campitiello, Pasqualina Rubini and Luca Gentilucci*

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The reaction of sulfonyl peptides containing L- or D-configured Ser or Thr with bis(succinimidyl) carbonate in the presence of a catalytic amount of a base affords, in solution or in the solid phase, the corresponding peptides with one or two, consecutive or alternate oxazolidin-2-ones (Oxd). The Oxd ring can be regarded to as a pseudo-Pro with an exclusively *trans* conformation of the preceding peptide bond; homochiral Oxd-containing peptides adopt extended conformations, while the presence of a D-configured Oxd favours folded conformations.

Introduction

Determining the receptor-bound structure of biologically active peptides is fundamental in drug design. However, the direct investigation of ligand–receptor complexes has met with considerable practical obstacles; besides, many native peptides are highly flexible molecules, therefore their conformational analysis is a difficult task. As a consequence, many efforts have been dedicated to the design of conformationally defined peptidomimetics as tools for investigating the key structural and conformational features on which receptor recognition and binding are based.[1]

Generally, peptide backbones serve as 3D scaffolds for positioning the side chains involved in ligand–receptor interactions. The presence of Pro residues strongly impacts upon the structural and conformational properties of the backbones. The cyclic structure of Pro forces the ϕ angle to $-65° \pm 15°$, thus preventing the formation of an α-helix, and promoting the formation of β-turns.[2] Turns and inverse turns play an important role in the architecture and bioactivity of native folded proteins.[3] In addition, while the barrier to secondary amide *cis*/*trans* isomerization is *ca.* 10 kcal mol^{-1} (Fig. 1), the presence of Pro reduces the barrier to just 2 kcal mol^{-1}, increasing the *cis* conformer.[4]

Besides Pro itself, numerous derivatives were found in proteins of microbial or marine origins, in antibiotics or cytotoxic peptides,[5] and many others (*e.g.* 2,4-MePro, Δ^3Pro, Azi, Aze, Pip, Nip, *etc.*, Fig. 1)[6] were synthesized and utilized to design conformationally constrained peptidomimetics.[1,7]

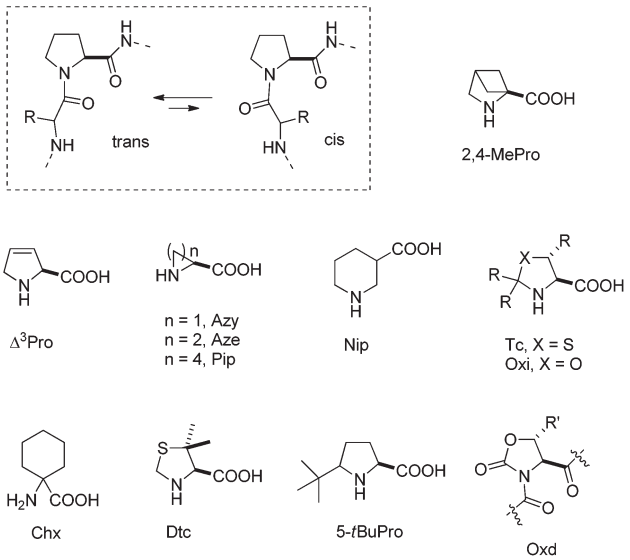


Fig. 1 Selected examples of pseudo-Pro structures.

For instance, pseudo-Pro such as thiazolidine- and oxazolidine-4-carboxylic acid (Tc, Oxi) dimethylated at the 2 position are known to be quantitative, or nearly quantitative, inducers of the *cis* conformation around the preceding peptide bond,[8] while 1-aminocyclohexane-1-carboxylic acid (Chx) induces the *trans* conformation.[9] On the other hand, 5,5-dimethylthiazolidine-4-carboxylic acid (Dtc)[10] and azetidine-2-carboxylic acid (Aze)[11] (Fig. 1) favor angles in the γ-turn region, while 5-*tert*-butylproline (5-*t*BuPro) stabilizes type VI β-turn conformation.[12]

Finally, pseudo-Pro have found use in the preparation of foldamers,[13] short synthetic oligomers which have a tendency to form

Department of Chemistry "G. Ciamician", University of Bologna, via Selmi 2, 40126 Bologna, Italy. E-mail: luca.gentilucci@unibo.it; Fax: +39 0512099456; Tel: +39 0512099570

† Electronic supplementary information (ESI) available: ^{1}H NMR analyses in different solvents; VT-^{1}H-NMR $\Delta\delta/\Delta t$ values in different solvents; non-obvious ROESY cross-peaks in 8 : 2 DMSO-d_6–H_2O; structures and energies of the intermediates estimated by semi-empirical computations for the cyclization reaction; CD spectra. See DOI: 10.1039/c2ob07172j

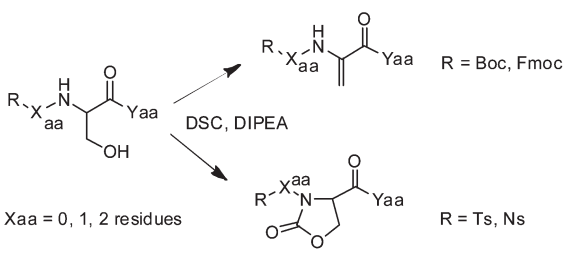
well-defined secondary structures, stabilized by noncovalent interactions.[14]

In recent years, we have been interested in the use of D-Pro, β-Pro, pseudo-Pro and pseudo-β-Pro for the preparation of constrained bioactive peptidomimetics.[15] In this regard, we recently proposed the 2-oxo-1,3-oxazolidine-4-carboxylate (or oxazolidin-2-one, in short: Oxd) as a constrained pseudo-Pro residue. Indeed, the carbonyl group of the cycle introduces a constraint that enforces the pseudo-peptide bond to always have the *trans* conformation (Fig. 1).

Apart from the different applications in medicinal chemistry,[16] Oxd-peptides have been the subject of much interest as foldamers or as self-assembling scaffolds forming nanostructures. The oligomers of the Boc-(L-Ala-D-Oxd)$_n$-OBn series exhibit a strong tendency to form helices with 10-membered H-bonded rings in solution.[17] The α,β-hybrid oligomers of the Boc-(L-β^3-hPhg-D-Oxd)$_n$-OBn series formed helices with 11-membered H-bonded turns for $n \geq 5$. The Boc-(L-β^3-hPhe-D-Oxd)$_n$-OBn series displays chain length-dependent behaviour. In the higher oligomers ($n > 2$), there is a stronger tendency to form intramolecular H-bonds, while the Boc-(L-β^3-hPhe-D-Oxd)$_2$-OBn motif forms an anti-parallel β-sheet-like structure, where only one intermolecular H-bond stabilizes the fibre-like material, as well as the hydrophobic forces between the aromatic side-chains.[18]

In this work we considerably expand the scope of our preliminarily described preparation of peptides containing the Oxd ring.[19] The reaction proceeds by the direct cyclization of *N*-sulfonyl peptides containing a Ser residue (Scheme 1) by treatment with bis(succinimidyl) carbonate (DSC) and diisopropylethylamine (DIPEA).

The distance between the sulfonyl and the Ser in the sequence is relatively unimportant. Indeed, the reaction of peptides having the sulfonyl group directly connected to the Ser, or separated by one or two amino acids (Scheme 1, Xaa = 0, 1, or 2 residues) proceeded with similar results. The reaction of the corresponding Fmoc- or Boc-peptides under the same conditions gave elimination to dehydroalanine (Dha), in agreement with the literature (Scheme 1).[20]



Scheme 1 Different reactivity of *N*-carbamate and *N*-tosyl (Ts) or nosyl (Ns) oligopeptides.

Results and discussion

Optimization of the reaction conditions

The reaction depicted in Scheme 1 represents the first synthesis of a oxazolidin-2-one-4-carboxylate directly within a peptide sequence. Apparently, the reaction outcome depends on the presence of the sulfonyl group (see Introduction). Subsequently, in order to optimize the cyclization of these sulfonyl peptides, we investigated the role of the carbonate, solvent, base, and sulfonyl group, by reacting the model peptides **1a–d** under different conditions (Scheme 2).

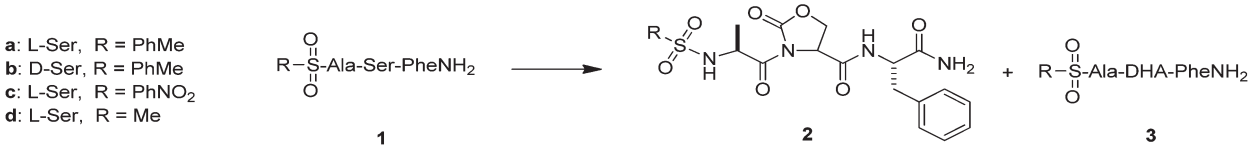
The preparation of **1a–d** and of the other sulfonyl-protected peptides **4**, **6**, **8**, **11** and **13**, was conducted by coupling the amino acids under normal conditions, using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC–HCl) and 1-hydroxybenzotriazole hydrate (HOBt) as activating agents (Table 1). *N*-Mesyl (Ms), tosyl (Ts), or nosyl (Ns) amino acids were prepared according to the literature.[21]

The treatment of **1a** (Table 2) with 1.2 equiv. of DSC and 1.2 equiv. of DIPEA in DCM–CH_3CN[20] gave the oxazolidinone-peptide **2a**, Ts-Ala-Oxd-PheNH$_2$ (entry 1) in good yield, together with traces of the corresponding elimination product Ts-Ala-Dha-PheNH$_2$ (**3**). The substitution of CH_3CN with DMF allowed an increase of the yield while reducing the reaction time (entry 2); the use of solely DMF or DMSO (entries 3 and 4) was not beneficial. Different bases such as DBU (entry 5) or DMAP (entry 6) in DCM–DMF gave **2** with comparable yields.

A second set of reactions was designed to test the role of the carbonate. The synthesis of an oxazolidin-2-one ring from an aminoalcohol and a carbonate or a dicarbonate is well documented in the literature.[22] Nevertheless, none of the procedures attempted by us gave the oxazolidinone-peptide **2** in a significant yield. The reaction of **1a** with 1,1′-carbonyldiimidazole (CDI) and DIPEA[23] gave Ts-Ala-Dha-PheNH$_2$ (**3**) as the major product, and only traces of **2a** (entry 7), the rest being the reagent. The treatment of **1a** with Boc_2O and DIPEA (entry 8) or DMAP[24] (entry 9) gave a Boc-derivative of **1** (not isolated) and traces of **2**, as determined by the HPLC-MS analysis of the reaction mixture. Triphosgene gave **2** and **3** only in traces (entry 10), while ethylchloroformate was completely ineffective (entry 11).[17,22,25]

Furthermore, we observed that the reaction of **1a** and DSC in the absence of a base did not furnish **2a** (entry 12). However, the reaction was possible with a catalytic amount of DIPEA (entry 13) or DMAP (entry 14), giving **2a** in excellent yield and in reasonable time in a DCM–DMF mixture, but not in DCM alone (entry 15).

By using DSC and a catalytic amount of DIPEA, Ts-Ala-D-Ser-PheNH$_2$ (**1b**) was converted into Ts-Ala-D-Oxd-PheNH$_2$ (**2b**) with the same yield of **2a** (entry 16 compared to entry 13).



Scheme 2 Reactions of the sulfonyl-peptides **2a**, **c**, **d**, containing L-Ser, and of **2b**, containing D-Ser, under the conditions reported in Table 2.

In order to gain more information about the role of the sulfonyl group in the cyclization, we compared the reaction of the Ts-peptide **1a** to that of the peptides **1c** and **1d** carrying the nosyl and mesyl group, respectively.

Under the same conditions as entry 13, Ns-Ala-Ser-PheNH$_2$ (**1c**) gave the corresponding Oxd-peptide Ns-AlaOxd-Phe-NH$_2$ (**2c**) in very good yield (entry 17).

On the contrary, the mesyl group failed to promote the cyclization. The treatment of Ms-Ala-Ser-PheNH$_2$ (**1d**) with DSC in the presence of a catalytic amount of DIPEA did not give rise to the formation of the Oxd-peptide (entry 18); the use of 1.2 equiv. of DIPEA (entry 19) or DMAP (entry 20) afforded the product only in traces.

Taken together, the results reported in Table 2 give several clues on the mechanism of the cyclization process. While an accurate investigation of the reaction mechanism is beyond the scope of this work, we compared the elimination of Boc- or Fmoc-peptides containing a Ser with a plausible mechanism of cyclization of the corresponding tosyl peptides (Scheme 3). The two reactions proceed *via* a common Ser-*O*-carbonate intermediate **A**. The elimination of the Boc- or Fmoc-intermediate **A** induced by the base (:B) gives Dha, as sketched in pathway **a**.

The alternative cyclization to Oxd was investigated with the aid of theoretical computations performed employing *ab initio* molecular orbital (MO) theory at the HF/6-31G** level. These

Table 1 RP-HPLC and ES-MS analyses of the linear peptides

Compd	ES-MS *m/z* [M + 1] *vs.* calcd	Purity[a] (%)	Compd	ES-MS *m/z* [M + 1] *vs.* Calcd	Purity[a] (%)
1a	477.2/477.1	89	**1b**	477.1/477.1	86
1c	508.2/508.2	86	**1d**	401.1/401.1	87
4	565.1/565.2	85	**6**	346.0/346.1	88
8	361.1/361.1	87	**11a**	374.1/374.1	90
11b	374.1/374.1	88	**13**[b]	—[c]	—[c]

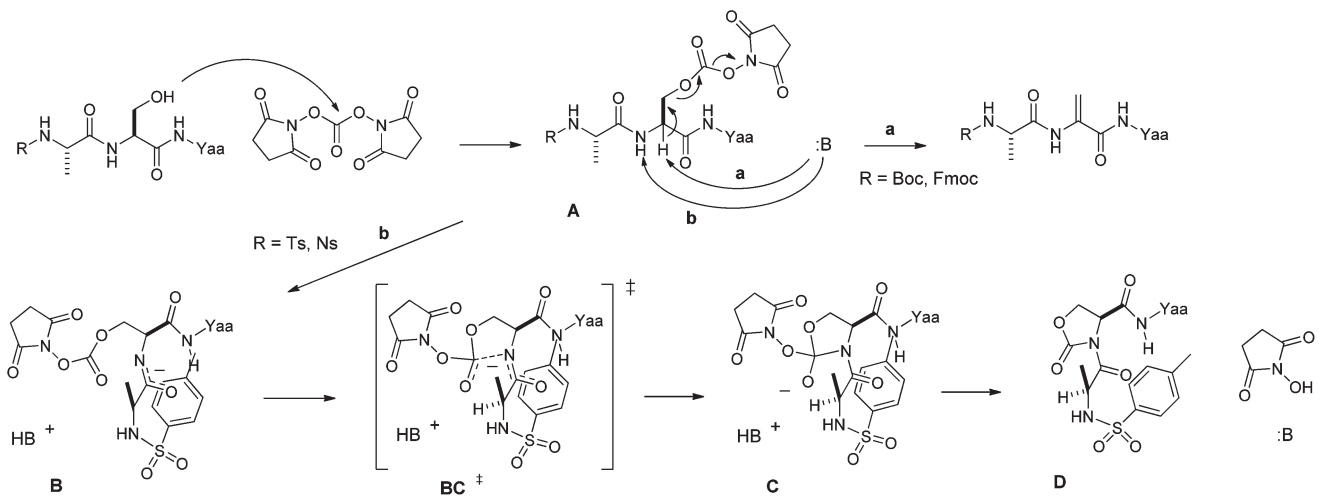
[a] Determined by analytical RP-HPLC, see General methods. [b] Ts-Ser-Phe-Ser-Phe-Wang; the following cyclization was performed in solid phase. [c] Not determined.

led to the proposal of the plausible reaction pathway **b**, which accounts for the experimental observations reported in Table 2. Upon treatment with :B, the Ts- or Ns-intermediate **A** would form the anionic intermediate **B**, which in turn gives rise to the five-membered anionic intermediate **C**. The loss of 2,5-dioxopyrrolidin-1-olate from **C** leads to the Oxd-peptide **D**; the protonation of the anionic leaving group by HB$^+$ would regenerate the free base, which can be utilized in catalytic amount.

The optimized conformations of **A–D** and the estimated ΔE are shown in the ESI, Scheme S1.† The most stable conformation of the anionic intermediate **B** shows the aromatic ring of the sulfonyl group stacked below the delocalized anion at a distance of 3.5 Å; this distance is perfectly compatible with the values reported in the literature for π-stacking interactions.[26] The ability of arylsulfonamido groups to form sandwich structures by π-stacking interactions is well described in the literature.[27] Besides, electron-poor aromatic rings effectively promote the reactivity of delocalized anions such as enolates by means of a donor–acceptor π-stacking stabilization of the transition states.[28] In a similar way, it is plausible that sulfonamido groups such as tosyl and nosyl, but not mesyl, might stabilize the intermediate anion **B** as well as the plausible transition state **BC** (Scheme 3). This effect would be impossible for Boc- or Fmoc-peptides; consequently, under the same conditions, these peptides undergo elimination to Dha.

Interestingly, the calculated structures of **C** and **D** clearly revealed an AlaCHα···$^{-1}$O–C interaction, and a non-conventional AlaCHα···O═C(Oxd) hydrogen bond, respectively. The latter interaction was also confirmed by NMR analysis and modeling of the Oxd-peptides (see next paragraph).

Starting from the calculated structures **A–D**, the dioxopyrrolidin-1-olyl group was replaced by other leaving groups resulting from CDI, Boc$_2$O, chloroformate, or triphosgene (see Table 2), and these new structures were optimized (not shown). The comparison revealed that the anionic intermediate **C** carrying the dioxopyrrolidin-1-olyl is significantly lower in energy with respect to the analogues carrying the other leaving groups. This comparatively higher stability might also reflect the transition state **BC**, accounting for the efficacy of DSC in the cyclization,



Scheme 3 Plausible reaction pathways for the elimination of carbamate-peptides (pathway **a**) and alternatives for the cyclization of arylsulfonyl peptides (pathway **b**).

Table 2 Reagents and conditions tested for the synthesis of R-SO_2-Ala-Oxd-PheNH_2 (**2**) from R-SO_2-Ala-Ser-PheNH_2 (**1**)

Entry	Compd	Carbonate (equiv.)	Base (equiv.)	Solvent	Time[a] (h)	**2**[b] (%)	**3** (%)
1	**a**	DSC (1.5)	DIPEA (1.2)	DCM–CH_3CN 3 : 1	2	74	traces[c,d]
2	**a**	DSC (1.5)	DIPEA (1.2)	DCM–DMF 3 : 1	1	88	—
3	**a**	DSC (1.5)	DIPEA (1.2)	DMF	1	80	—
4	**a**	DSC (1.5)	DIPEA (1.2)	DMSO	1	79	—
5	**a**	DSC (1.5)	DBU (1.2)	DCM–DMF 3 : 1	2	85	—
6	**a**	DSC (1.5)	DMAP (1.2)	DCM–DMF 3 : 1	3	87	—
7	**a**	CDI (1.2)	DIPEA (1.2)	DCM	24	Traces[c,d]	30[d,e]
8	**a**	Boc_2O (2.1)	DIPEA (1.2)	CH_3CN	24	—[d,e,f]	—
9	**a**	Boc_2O (2.1)	DMAP (1.2)	DCM–DMF 3 : 1	2	—[d,e,f]	—
10	**a**	$Cl_3COCOOCCl_3$ (0.5)	DIPEA (3.0)	DCM–DMF	24	—	traces[c,d]
11	**a**	ClCOOMe	DIPEA (1.2)	DCM–DMF 3 : 1	24	—[d,e,g]	—
12	**a**	DSC (1.2)	—	DCM–DMF 3 : 1	24	—	—
13	**a**	DSC (1.2)	DIPEA (0.1)[h]	DCM–DMF 3 : 1	3	92	—
14	**a**	DSC (1.2)	DMAP (0.3)[h]	DCM–DMF 3 : 1	6	90	—
15	**a**	DSC (1.2)	DIPEA (0.1)[h]	DCM	24	Traces[c,d,e]	—
16	**b**	DSC (1.2)	DIPEA (0.1)[h]	DCM–DMF 3 : 1	3	92	—
17	**c**	DSC (1.2)	DIPEA (0.1)[h]	DCM–DMF 3 : 1	3	90	—
18	**d**	DSC (1.2)	DIPEA (0.1)[h]	DCM–DMF 3 : 1	24	—	—
19	**d**	DSC (1.5)	DIPEA (1.2)	DCM–DMF 3 : 1	24	Traces[c,d,e]	Traces[c,d,e]
20	**d**	DSC (1.5)	DMAP (1.2)	DCM–DMF 3 : 1	24	Traces[c,d,e]	Traces[c,d,e]

[a] The reaction was stopped at the disappearance of the reagent, as determined by the t.l.c. analysis of the reaction, or after 24 h. [b] After purification by flash chromatography. [c] <5%. [d] Not isolated, determined by the HPLC-MS analysis. [e] The rest being the reagent **1**. [f] Boc-**1** was the mayor by-product. [g] MeOCO-**1** was the major by-product. [h] The lower amount tested.

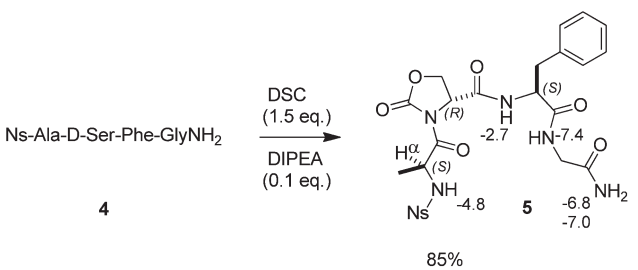
compared to the other carbonates or dicarbonates, *etc.*, discussed in Table 2, entries 8–11.

Finally, it is plausible that the π-stacking is functional also at stabilizing the anionic intermediate of type **B** for peptides having the arylsulfonamido group and Ser separated by two amino acids[19] (not calculated).

Epimerization during the cyclization process was excluded on the basis of the comparison of the NMR and HPLC analyses of **2a** *vs.* **2b**, including the HPLC analysis on a chiral stationary phase (see General methods).

The mild cleavage of the arylsulfonyl groups[29] was discussed previously.[19] The tosyl group was removed in sufficient yield with iodotrimethylsilane,[30] while the treatment with SmI_2–pyrrolidine–water[31] was much less efficient. The cleavage of the Ns group was easily performed with K_2CO_3–PhSH,[32] giving the deprotected peptide in good yield.

In summary, the comparison of the results listed in Table 2 led us to conclude that the conditions of entry 13 were the best for the cyclization reaction. Consequently, the Oxd-tetrapeptide Ns-Ala-D-Oxd-Phe-GlyNH_2 (**5**), useful for the investigation of the conformational features of the constrained peptides, was smoothly prepared from Ns-Ala-D-Ser-Phe-GlyNH_2 (**4**) by treatment with DSC and 0.1 equiv. of DIPEA in 3 : 1 DCM–DMF (Scheme 4).

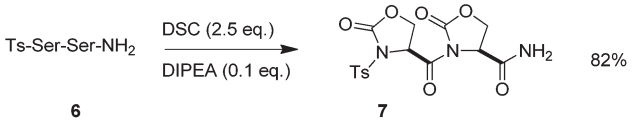


Scheme 4 Synthesis of **5** and $\Delta\delta/\Delta t$ VT-NMR values of the amide protons in 8 : 2 DMSO-d_6–H_2O.

Synthesis of di-Oxd-peptides

Previous results[19] demonstrated that the position of a Ser or Thr residue in the sequence of the sulfonyl-peptide is practically unimportant for the cyclization to Oxd-peptide (see above). As a consequence, we envisaged the opportunity to perform a single-step cyclization of sequences containing two Ser, or a Ser and a Thr, consecutive or alternating with other amino acids.

The reaction of Ts-Ser-SerNH_2 (**6**) with 2.5 equiv. of DSC in 3 : 1 DCM–DMF and in the presence of a catalytic amount of DIPEA (0.1 equiv.) gave the corresponding di-Oxd-amide **7** in excellent yield (Scheme 5) after isolation by flash chromatography.

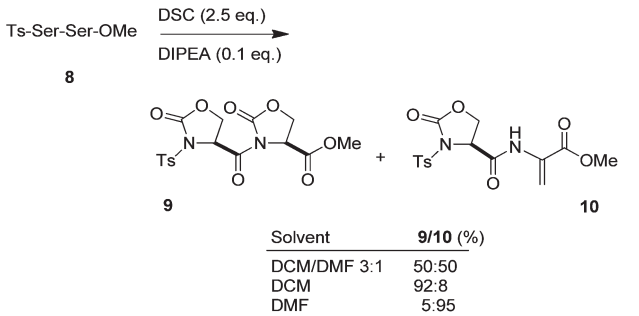


Scheme 5 Synthesis of the di-Oxd peptide amide **7**.

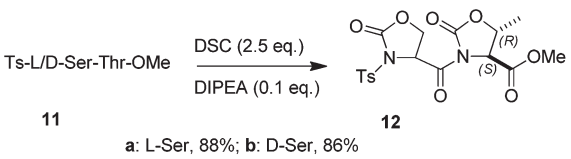
On the other hand, the reaction of the dipeptide ester Ts-Ser-SerOMe (**8**) under the same reaction conditions gave a *ca.* 1 : 1 mixture of the expected di-Oxd peptide **9** and the dipeptide containing the Dha methyl ester **10** (Scheme 6).

Possibly, this result reflects the comparatively higher acidity of the Hα of serine methyl ester with respect to that of the serine amides,[33] which promotes the elimination of the intermediate Ser-*O*-succinimidyl carbonate.

Interestingly, the reaction outcome was dependent on the solvent utilized (Scheme 6). Indeed, performing the reaction in DCM gave Ts-Oxd-Oxd-OMe (**9**) and Ts-Oxd-Dha-OMe (**10**, Dha = dehydroalanine) in 92 : 8 ratio (88% overall yield), while in DMF the situation was completely reversed, giving a 95 : 5 ratio in favor of **10** (86% overall yield).



Scheme 6 Solvent effect in the alternative formation of the di-Oxd peptide **9** and Oxd-Dha peptide **10**.



Scheme 7 Synthesis of the di-Oxd peptide esters **12a**, **b**.

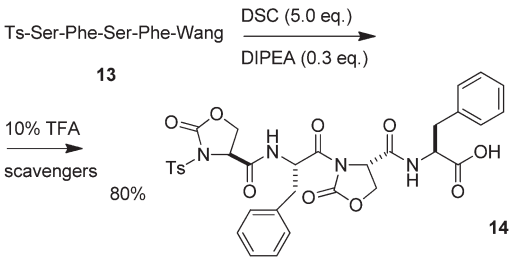
This dipeptide Ts-Oxd-Dha-OMe is of some interest, since it contains two distinct secondary structure-inducing residues, the Oxd and a dehydroamino acid. Indeed, dehydroamino acids are well known inducers of β-turn structures; for instance, sequential placement of dehydroPhe (ΔPhe) in oligomers gives repeated β-turns forming 3_{10} helices.[20,34]

In a similar way as for the preparation of **9**, the cyclization of the peptide esters **11a**, **b** containing L-Ser, Thr, and D-Ser, Thr, respectively, gave in DCM the di-Oxd **12a**, **b** in high yield (Scheme 7), with only traces of the elimination products.

The *trans* configuration of the 5-methyl-oxazolidin-2-one-4-carboxylate was deduced by the ^{1}H NMR coupling constant of the H4–H5 (*J ca.* 5.0 Hz), thus retaining the (2*S*,3*R*) stereochemistry of Thr. The comparison of the NMR and HPLC analyses of **12a** *vs.* **12b**, including the HPLC analysis on a chiral stationary phase (see General methods) led us to consider that epimerization was negligible.

Finally, the di-Oxd-tetrapeptide acid Ts-Oxd-Phe-Oxd-Phe-OH (**14**) was entirely synthesized on solid-phase (Scheme 8). The resin-bound precursor Ts-Ser-Phe-Ser-Phe-Wang (**13**) was prepared under standard conditions, and was subjected to cyclization by treatment with a 5 equiv. excess of DSC and catalytic DIPEA (0.3 equiv.) prior to the cleavage from the resin.

The cleavage of the di-Oxd-peptide was performed by two consecutive treatments with 10% TFA in DCM in the presence of scavengers. The peptide **14** was isolated by semi-preparative RP-HPLC with a very satisfactory yield.



Scheme 8 Solid-phase synthesis of **14**.

Conformational aspects of the Oxd peptides

In order to analyze the conformational bias exerted by the Oxd on the overall structure of the peptides, we performed the conformational analyses of the representative examples **2a**, **2b**, **5**, **10**, containing one Oxd, and of **12a**, **12b**, **14**, which contain two consecutive or alternate Oxd rings.

Electronic Circular Dichroism (ECD) is a widely used technique for studying protein and peptide conformations. This technique is intrinsically a low-resolution method; however, it can furnish qualitative information on the presence of ordered secondary structures,[35] although not too many examples on short peptides are reported.[36] ECD measurements of compounds **2a**, **2b**, **5**, **10**, **12a**, **12b** and **14** were performed at room temperature in TFE[37] and methanol; **10** and **12a** also in chloroform (ESI, Fig. S2†). These analyses gave some information on the eventual occurrence of secondary structures. Spectra of **2b**, **5**, **10** and **12b** show a negative nπ* band at *ca.* 235 nm, compatible with γ-turn structures.[38] Spectra of structurally correlated **2a** **2b**, and of **12a** **12b** show marked differences. CD intensity changes when moving from methanol (and chloroform for **10**) to TFE, indicating that the nature of the solvent may influence the turn population. Different chiroptical properties are associated to the other compounds **2a**, **12a**, **14** in the same spectral region. For instance, by comparing ECD spectra of **12a** with those of **12b**, a specular relationship emerges in the nπ* region, possibly reflecting the inversion of configuration of Oxd[1]. CD spectra of **12a** present a positive band centered at *ca.* 230 nm accompanied by a weak positive shoulder at lower energy, while a negative ππ* band is observed at around 200 nm. In both cases the magnitude of CD grows when moving from TFE to methanol and chloroform. These spectral features suggest that for **12a**, a different secondary structure becomes predominant with respect to the one characterizing **12b**, and its stability is increased in chloroform.

The Oxd-peptides were analyzed by NMR experiments using standard techniques at 400 MHz in $CDCl_3$, CH_3OH, and in the biomimetic medium 8 : 2 DMSO-d_6–H_2O.[39] For most compounds, the ^{1}H NMR resonances of the compounds showed modest variations of the chemical shifts in the different solvents, suggesting conformational stability (exceptions are discussed throughout).

It has been demonstrated that the Oxd confers the preceding amide bond a well defined *trans* conformation.[17,18] Accordingly, the ^{1}H NMR analyses of all of the Oxd-peptides showed a single set of sharp resonances in each solvent, indicating conformational homogeneity or a fast equilibrium between conformers; generally, linear peptides containing a Pro show two sets of resonances, for the *cis* and *trans* conformers (see Introduction).

The ^{1}H NMR analyses of all of the compounds showed a significantly downfield position of the Hα proton of the residue preceding the Oxd. For instance, for the AlaHα in Ts-Ala-Oxd-PheNH$_2$ (**2a**) it is at *ca.* 5.1 ppm, while in Ts-Ala-Ser-PheNH$_2$ (**1a**) it is at *ca.* 3.5 ppm; in Ts-Oxd^1-Oxd^2-OMe (**9**), the Oxd^{1}H4 is at *ca.* 6.0 ppm, while Oxd^{2}H4 is at *ca.* 5.0 ppm. This accounts for a strong deshielding effect exerted by Oxd(C═O),[17,18] compatible with a non-conventional CH···O═C intramolecular

hydrogen bond,[40] and confirms the *trans* conformation of the amide bond between the Oxd and the preceding, deshielded residue.

Variable temperature (VT)-^{1}H-NMR experiments were utilized to deduce the presence of H-bonds (Tables S1–3, ESI†). The homochiral Oxd-peptide Ts-Ala-Oxd-PheNH$_2$ (**2a**) did not manifest the presence of any H-bond: the $\Delta\delta/\Delta t$ values of AlaNH, PheNH in 8 : 2 DMSO-d$_6$–H$_2$O were −6.8, and −4.3 ppb K^{-1}, respectively.

On the other hand, for Ts-Ala-D-Oxd-PheNH$_2$ (**2b**) the $\Delta\delta/\Delta t$ (ppb K^{-1}, 8 : 2 DMSO-d$_6$–H$_2$O) values of AlaNH, PheNH were −4.2, and −2.6, and for Ts-Ala-D-Oxd-Phe-GlyNH$_2$ (**5**) the $\Delta\delta/\Delta t$ (ppb K^{-1}, 8 : 2 DMSO-d$_6$–H$_2$O) values of AlaNH, PheNH, and GlyNH were −4.8, −2.7, and −7.4. The comparatively lower VT-^{1}H-NMR $\Delta\delta/\Delta t$ parameters of PheNH for **2b** and **5** are suggestive of the occurrence of a significant amount of folded conformations having PheNH involved in a H-bond ($|\Delta\delta/\Delta t| \lesssim$ 2.5 ppb K^{-1}).[32,41] The same trends can be observed also in the other solvents (Table S1, ESI†).

Molecular backbone conformations were investigated by 2D ROESY analysis in 8 : 2 DMSO-d$_6$–H$_2$O at 400 MHz, and the intensities of the resulting cross-peaks were ranked to infer plausible interproton distances (Tables S4–10, ESI†). 2D gCOSY experiments were utilized for the unambiguous assignment of all of the resonances.

Structures consistent with the spectroscopic analyses were obtained by restrained MD simulations, using the distances derived from ROESY as constraints, and minimized with the AMBER force field. The ω bonds were set at 180°, as the absence of Hαi–H$\alpha(i+1)$ cross-peaks excluded *cis* peptide bonds. Simulations were conducted in a box of explicit, equilibrated TIP3P water molecules. Random structures were generated by unrestrained high-temperature MD; the structures were subjected to high-temperature restrained MD with a scaled force field, followed by a simulation with full restraints. Finally, the system was gradually cooled, and the structures were minimized. The results were clustered by the RMSD analysis of the backbone atoms.

For Ts-Ala-Oxd-PheNH$_2$ (**2a**) and Ts-Ala-D-Oxd-PheNH$_2$ (**2b**), the procedure gave one major cluster each, comprising *ca.* 80% of the structures. For each compound cluster, the representative geometries with the lowest internal energy were selected and analyzed (Fig. 2). The comparison of the two structures shows a clear difference; while **2a** adopts an extended conformation, the peptide containing the D-Oxd shows a preference for a folded conformation, compatible with a γ-turn centered on D-Oxd.

To investigate the dynamic behavior of **2a** and **2b**, the structures were analyzed by unrestrained MD for 10 ns in a box of explicit water molecules. Besides the different random conformations, the analysis of the trajectories of **2b**, but not of **2a**, revealed a well-defined γ-turn secondary structure stabilized by an explicit H-bond between PheNH and the Ala(C═O), in agreement with the VT-NMR parameters.

For Ns-Ala-D-Oxd-PheGly-NH$_2$ (**5**), the conformational analysis gave two clusters comprising altogether more than 85% of the structures. For each cluster, the representative geometries **5A** and **5B** with the lowest internal energy were selected and analyzed (Fig. 2). They differ almost exclusively by the opposite orientation of the D-Oxd-Phe peptide bond. Possibly, the two structures represent conformers in fast equilibrium. The structure **5A** shows a clear inverse γ-turn centered on D-Oxd, and is

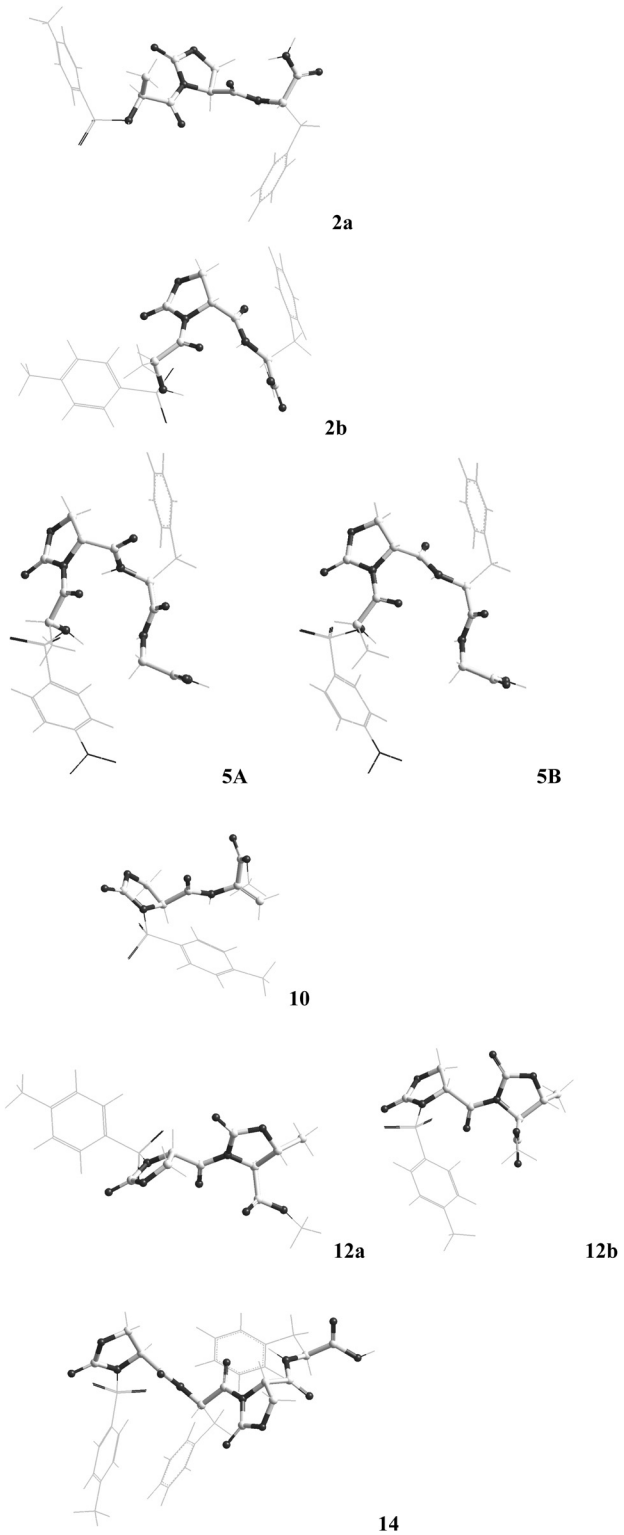


Fig. 2 Representative low-energy structures of **2a**, **2b**, **5** (**A**, **B**), **10**, **12a**, **12b**, **14** consistent with ROESY analysis, calculated by restrained MD in a 30 × 30 × 30 box of standard TIP3P water molecules. Backbones and Oxd are rendered in balls and cylinders, the rest in sticks.

ca. 1.4 kcal mol^{-1} lower in energy than **5B**; the latter shows some violations of the distance constraint involving PheNH.

To investigate the dynamic behavior of **5**, the two conformers **A** and **B** were analyzed by unrestrained MD for 10 ns in a box of standard TIP3P water molecules. The simulation showed the conversion of one conformation into the other. The analysis of the trajectories of **5A** revealed the occurrence of well-defined secondary structures stabilized by an explicit H-bond between Ala(C═O) and PheNH, in agreement to the VT-NMR parameters. On the other hand, the analysis of the trajectories of **5b** revealed that occasionally the structure adopts an inverse β-turn type I conformation, stabilized by a H-bond between GlyNH and the Ala(C═O), but this observation is not supported by the VT-NMR analysis.

The preference for the folded structures shown by **2b** and **5** makes sense. It is well known that linear oligopeptides including a heterochiral Pro show higher propensity to adopt stable inverse γ- or β-turns, the Pro occupying the position $i + 1$, compared to the peptides composed of all L-amino acids.[42] Besides, 10- and 7-membered H-bonded rings form competitively to each other, depending on the solvent,[43] on the prevalence of a *trans* over *cis* conformation of the amide bond preceding Pro,[44] and/or on the nature and steric hindrance of the amino acids preceding and following Pro.[45]

The conformational analysis of (**10**) gave one cluster comprising the large majority of the structures. The representative geometry with the lowest internal energy is shown in Fig. 2. The calculated geometry of the Dha residue perfectly matches the structures reported in the literature.[46]

The conformational preference of **10** showed some dependence on the solvent. The ^{1}H NMR resonances of the H4 and H5 protons of Oxd in $CDCl_3$ showed significant differences with respect to 8 : 2 DMSO-d_6–H_2O and CH_3OH.

The VT-NMR analysis in $CDCl_3$ gave DhaNH $\Delta\delta/\Delta t = -1.8$ ppb K^{-1}, and in 8 : 2 DMSO-d_6–H_2O $\Delta\delta/\Delta t = -4.6$ ppb K^{-1}. Apparently, in the less competitive $CDCl_3$ the DhaNH is involved in a H-bond with one of the S═O, conferring on **10** a pseudo-γ-turn structure, which is not observed in 8 : 2 DMSO-d_6–H_2O. Accordingly, the unrestrained molecular dynamics simulations for 10 ns in explicit water did not show this pseudo-γ-turn conformation.

The ROESY-restrained MD simulations of Ts-Oxd^1-(5-Me-Oxd^2)-OMe (**12a**) and Ts-D-Oxd^1-(5-Me-Oxd^2)-OMe (**12b**) gave, after clustering, one cluster each, comprising almost the totality of the structures; the representative ones are shown in Fig. 2. The two oxazolidin-2-ones are nearly orthogonal to each other. The unrestrained MD confirmed the rigidity of the conformations, since the rotation of the two Oxd rings one with respect to the other was not observed during the simulations.

The calculated conformation of the Ts-Oxd^1-Phe^2-Oxd^3-Phe^4-OH (**14**), determined as described above, presents an extended conformation (Fig. 2), confirming that homochiral peptides do not tend to fold.

The ^{1}H NMR resonances of the compound significantly vary in the different environments; for instance, the δ of Phe^{4}Hα in 9 : 1 $CDCl_3$–DMSO-d_6 (this compound is not soluble in pure chloroform), 8 : 2 DMSO-d_6–H_2O, and CH_3OH is 4.83, 4.53, 4.22, respectively, and the δ of Phe^{2}Hα is 5.78, 5.57, 5.68.

The VT-NMR analysis in 9 : 1 $CDCl_3$–DMSO-d_6 indicated some preference for a pseudo-γ-turn on Oxd^1 stabilized by a H-bond between Oxd^1(S═O) and Phe^{2}NH (Phe^{2}NH $\Delta\delta/\Delta t = -2.9$ ppb K^{-1}). However, as observed for **10**, this conformation is not stable in more polar environments (for instance, in 8 : 2 DMSO-d_6–H_2O Phe^{2}NH $\Delta\delta/\Delta t = -5.2$ ppb K^{-1}), and it is not observed during the unrestrained MD simulations in explicit water.

Conclusions

In this work we discuss our methodology for the straightforward preparation of Oxd-peptides starting from arylsulfonyl peptides containing L- or D-configured Ser or Thr by treatment with DSC and a base.

The mechanism was investigated by varying the reaction conditions, and the results were rationalized with the aid of theoretical computations. The experiments highlighted the role of the arylsulfonylamido group and DSC. Indeed, while tosyl and nosyl gave the ring closure, the mesyl group was ineffective; and CDI, Boc_2O, triphosgene and ClCOOMe failed to afford the Oxd-peptides in significant yield. The reaction was performed by using catalytic amounts of DIPEA, DBU, or DMAP.

Essentially, computations suggested that the electron-poor arylsulfonylamido group might effectively stabilize the anionic intermediate which leads to the Oxd ring by a π-stacking interaction. Computations also allowed the rationalization of the effectiveness of DSC compared to other carbonates or dicarbonates.

We expanded the scope of the methodology by preparing in a single step di-Oxd-peptides from peptides containing two consecutive Ser, or Ser and Thr, or two Ser separated by other amino acids. The peptide amide gave the corresponding di-Oxd-amide, as expected, while peptide methyl ester gave the di-Oxd-peptide or the peptide Oxd-Dha, depending on the solvent selected for the reaction. The synthesis of the linear precursors and the cyclization reaction was performed either in solution or in the solid phase, making the entire process a convenient method for the preparation of constrained peptides or foldamers.

These Oxd residues can be regarded to as suitable constrained pseudo-Pro. The peptides containing the Oxd in place of Pro show an all-*trans* conformation instead of mixtures of *cis* and *trans* conformers. Homochiral sequences tend to adopt extended conformations, while the presence of a D-Oxd ring induces folded conformations, with an inverse γ-turn centered on D-Oxd, stabilized by an explicit H-bond.

Experimental

General methods

Unless stated otherwise, standard chemicals were obtained from commercial sources and used without further purification. Flash chromatography was performed on silica gel (230–400 mesh), using mixtures of distilled solvents. Analytical RP-HPLC was performed on an ODS column (4.6 μm particle size, 100 Å pore diameter, 250 μm, DAD 210 nm, from a 9 : 1 H_2O–CH_3CN to a 2 : 8 H_2O–CH_3CN in 20 min) at a flow rate of 1.0 mL min^{-1}, followed by 10 min at the same composition. Semi-preparative RP-HPLC was performed on a C18 column (7 μm particle size,

21.2 mm × 150 mm, from 8 : 2 H_2O–CH_3CN to 100% CH_3CN in 10 min) at a flow rate of 12 mL min^{-1}. Purities were assessed by analytical RP-HPLC under the above reported conditions and elemental analysis. Chiral HPLC analysis was performed on a CHIRALPAK IC column (0.46 cm × 25 cm), n-hexane–2-propanol 1 : 1, at 0.8 mL min^{-1}. Semi-preparative and analytical RP-HPLC of the peptide acid **14** was performed as reported above, with the addition of 0.1% TFA in the mobile phase. Elemental analyses were performer using a Thermo Flash 2000 CHNS/O analyzer. NMR spectra were recorded on a Varian instrument. Circular Dichroism (CD) spectra were recorded on a Jasco J-710 spectropolarimeter.

Peptide synthesis

A stirred solution of the *N*-protected amino acid in 4 : 1 DCM–DMF (5 mL) was treated with HOBt (1.2 equiv.), at r.t. and under inert atmosphere. After 10 min, the *C*-protected amino acid (1.1 equiv.), EDCI–HCl (1.2 equiv.) and TEA (3 equiv.) were added while stirring at r.t. under inert atmosphere. After 3 h, the mixture was concentrated at reduced pressure, and the residue was diluted with EtOAc (25 mL). The solution was washed with 0.1 M HCl (5 mL), and a saturated solution of $NaHCO_3$ (5 mL). The organic layer was dried over Na_2SO_4 and the solvent was evaporated at reduced pressure. The crude peptides were analyzed by HPLC-MS analysis, and were used without further purifications (Table 1).

The intermediate *N*-Boc peptides were deprotected by treatment with 1 : 2 TFA/DCM (5 mL), with stirring, at r.t. After 15 min, the solution was evaporated under reduced pressure, and the treatment was repeated. The residue was suspended in Et_2O (20 mL). The peptide-TFA salts which precipitated were used for the next couplings without further purifications.

Mono-Oxd-peptide synthesis

Ts-Ala-Oxd-Phe-NH_2 (2a). DSC (0.13 g, 0.50 mmol) was added to a stirred solution of the linear peptide Ts-Ala-Ser-Phe-NH_2 (**1a**, 0.20 g, 0.42 mmol), in 3 : 1 DCM–DMF (4 mL) followed by DIPEA (7.4 μL, 0.04 mmol) at r.t. and under inert atmosphere. After 3 h, the solvent was removed under reduced pressure, the residue was diluted with 0.1 M HCl (5 mL), and the mixture was extracted three times with DCM (5 mL). The combined organic layers were dried over sodium sulfate, filtered, and concentrated at reduced pressure. The residue was purified by flash chromatography over silica-gel (eluant 1 : 1 hexane–EtOAc, column size: 15 × 1.0 cm^2) to give **2a** (0.194 g, 92%, 95% pure by analytical RP-HPLC). IR (nujol) ν 1766, 1722, 1649, 1530 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.31 (d, J = 7.3 Hz, 3H, AlaMe), 2.39 (s, 3H, Ts*Me*), 3.01–3.16 (m, 2H, PheHβ), 4.26 (dd, J = 4.2, 9.3 Hz, 1H, OxdH5), 4.44 (t, J = 9.3 Hz, 1H, OxdH5), 4.63 (q, J = 7.4 Hz, 1H, PheHα), 4.90 (dd, J = 4.2, 8.6 Hz, 1H, OxdH4), 5.12 (dq, J = 7.4, 9.3 Hz, 1H, AlaHα), 6.03 (br.s, 1H, $CONH_2$), 6.35 (br.s, 1H, $CONH_2$), 6.40 (d, J = 9.3 Hz, 1H, AlaNH), 7.09–7.26 (m, 7H, ArH), 7.75 (d, J = 8.4 Hz, 2H, ArH), 7.96 (d, J = 8.0 Hz, 1H, PheNH); ^{13}C NMR (2 : 1 $CDCl_3$–DMSO-d_6) δ 18.5, 20.8, 39.1, 47.8, 50.7, 54.9, 56.0, 127.1, 127.3, 128.7, 129.4, 129.8, 136.4, 139.5, 143.9, 152.4, 167.9, 173.5, 174.5; RP-HPLC (see General methods) 7.01 min; ES-MS m/z 503.2 [M + 1], calcd 503.2; Elem. Anal. for $C_{23}H_{26}N_4O_7S$, calcd: C 54.97, H 5.21, N 11.15, S 6.38; found: C 54.92, H 5.19, N 11.10, S 6.36.

Ts-Ala-D-Oxd-Phe-NH_2 (2b).). The reaction of Ts-Ala-D-Ser-Phe-NH_2 (**1b**, 0.20 g, 0.42 mmol) under the same conditions as described for **1a** gave **2b** (0.190 g, 90%, 96% pure by analytical RP-HPLC). IR (nujol) ν 1770, 1717, 1651, 1528 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.25 (d, J = 7.2 Hz, 3H, AlaMe), 2.38 (s, 3H, Ts*Me*), 2.85 (dd, J = 9.2, 14.0 Hz, 1H, PheHβ), 3.25 (dd, J = 4.8, 14.0 Hz, 1H, PheHβ), 3.75 (dd, J = 3.0, 8.4 Hz, 1H, OxdH5), 4.28 (t, J = 9.2 Hz, 1H, OxdH5), 4.66 (q, J = 7.4 Hz, 1H, PheHα), 4.72 (dd, J = 3.6, 9.2 Hz, 1H, OxdH4), 5.09 (quint, J = 7.8 Hz, 1H, AlaHα), 6.00 (br.s, 1H, $CONH_2$), 6.62 (d, J = 9.2 Hz, 1H, AlaNH), 6.70 (br.s, 1H, $CONH_2$), 7.02–7.29 (m, 7H, ArH), 7.65 (d, J = 8.4 Hz, 2H, ArH), 8.23 (d, J = 8.4 Hz, 1H, PheNH); ^{13}C NMR (2 : 1 $CDCl_3$–DMSO-d_6) δ 19.0, 21.1, 40.1, 47.0, 50.2, 55.6, 55.8, 127.3, 127.5, 129.1, 129.5, 130.1, 136.7, 139.9, 143.1, 153.4, 168.1, 173.2, 173.5; RP-HPLC (see General methods) 7.01 min; ES-MS m/z 503.2 [M + 1], calcd 503.2; Elem. Anal. for $C_{23}H_{26}N_4O_7S$, calcd: C 54.97, H 5.21, N 11.15, S 6.38; found: C 54.94, H 5.24, N 11.09, S 6.34.

Ns-Ala-Oxd-Phe-NH_2 (2c). The reaction of Ns-Ala-Ser-Phe-NH_2 (**1c**, 0.20 g, 0.39 mmol) under the same conditions as described for **1a** gave **2c** (0.189 g, 90%, 96% pure by analytical RP-HPLC). IR (nujol) ν 1783, 1710, 1705, 1653, 1525 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.21 (d, J = 6.8 Hz, 3H, AlaMe), 1.33 (d, J = 7.2 Hz, 3H, AlaMe), 2.43 (s, 3H, Ts*Me*), 3.02 (dd, J = 6.4, 13.8 Hz, 1H, PheHβ), 3.06 (dd, J = 6.9, 13.8 Hz, 1H, PheHβ), 3.77 (quint, J = 7.3 Hz, 1H, AlaHα), 4.22 (dd, J = 4.3, 9.0 Hz, 1H, OxdH5), 4.44 (t, J = 9.2 Hz, 1H, OxdH5), 4.62 (q, J = 7.1 Hz, 1H, PheHα), 4.92 (dd, J = 4.3, 8.8 Hz, 1H, OxdH4), 5.27 (quint, J = 6.8 Hz, 1H, AlaHα), 5.79 (br.s, 1H, $CONH_2$), 6.51 (br.s, 1H, $CONH_2$), 6.61 (d, J = 8.4 Hz, 1H, AlaNH), 7.10–7.28 (m, 8H, ArH + AlaNH), 7.72 (d, J = 8.0 Hz, 2H, ArH), 7.80 (d, J = 7.2 Hz, 1H, PheNH); ^{13}C NMR (2 : 1 $CDCl_3$–DMSO-d_6) δ 16.3, 18.0, 20.5, 36.7, 47.1, 51.3, 53.6, 55.2, 64.8, 125.9, 126.1, 127.4, 128.4, 128.7, 135.6, 136.0, 142.6, 151.4, 166.8, 170.6, 171.7, 171.9; Elem. Anal. for $C_{22}H_{23}N_5O_9S$, calcd: C 49.53, H 4.35, N 13.13, S 6.01; found: C 49.49, H 4.33, N 13.13, S 5.98.

Ns-Ala-D-Oxd-Phe-Gly-NH_2 (5). The reaction of Ns-Ala-D-Oxd-Phe-Gly-NH_2 (**4**, 0.20 g, 0.35 mmol) under the same conditions as described for **1a** gave **5** (0.178 g, 85%, 96% pure by analytical RP-HPLC). IR (nujol) ν 1768, 1719, 1655, 1534 cm^{-1}; ^{1}H NMR (9 : 1 $CDCl_3$–DMSO-d_6) δ 1.13 (d, J = 7.0 Hz, 3H, Me), 3.01 (dd, J = 6.2, 13.8 Hz, 1H, PheHβ), 3.23 (dd, J = 6.8, 13.8 Hz, 1H, PheHβ), 3.64 (dd, J = 3.1, 8.9 Hz, 1H, D-OxdH5), 3.70–3.79 (m, 2H, GlyHα), 4.30 (t, J = 8.6 Hz, 1H, D-OxdH5), 4.64 (q, J = 7.1 Hz, 1H, PheHα), 4.72 (dd, J = 2.9, 9.0 Hz, 1H, D-OxdH4), 5.13 (quint, 7.1 Hz, 1H, AlaHα), 6.62 (br.s, 1H, $CONH_2$), 7.05 (br.s, 1H, $CONH_2$), 7.11–7.25 (m, 5H, ArH), 7.90 (*d*, J = 8.4 Hz, 2H, ArH), 8.02 (t, J = 7.9 Hz, 1H, GlyNH), 8.29 (d, J = 8.0 Hz, 1H, PheNH), 8.35 (d, J = 8.4 Hz, 2H, ArH), 8.40 (d, J = 9.3 Hz, 1H, AlaNH); ^{13}C NMR (DMSO-d_6) δ 18.0, 37.6, 42.2, 51.6, 56.0, 58.2, 62.4, 125.2, 125.9, 127.8, 128.8, 128.6, 136.7, 150.4, 151.3, 153.9, 169.9, 171.7, 172.3, 173.2; RP-HPLC (see General methods) 7.85 min; ES-MS m/z 591.2 [M + 1], calcd 591.1; Elem. Anal. for

$C_{24}H_{26}N_6O_{10}S$, calcd: C 48.81, H 4.44, N 14.23, S, 5.43; found: C 48.77, H 4.47, N 14.19, S, 5.47.

Di-Oxd-peptide synthesis

Ts-Oxd^1-Oxd^2-NH$_2$ (7). To a stirred solution of Ts-Ser-Ser-NH_2 (**6**, 0.2 g, 0.58 mmol) in 3 : 1 DCM–DMF (4 mL), DSC (0.37 g, 1.45 mmol) and DIPEA (10 μL, 0.06 mmol) were added at r.t. under inert atmosphere. After 4 h, work up was performed as described above for **2a**, giving **7** (0.189 g, 82%, 95% pure by analytical RP-HPLC). IR (nujol) *ν* 1776, 1770, 1725, 1711; ^{1}H NMR ($CDCl_3$) *δ* 2.43 (s, 3H, Me), 4.37 (dd, *J* = 2.8, 9.2 Hz, 1H, Oxd^{1}H5), 4.48 (dd, *J* = 3.6, 9.6 Hz, 1H, Oxd^{2}H5), 4.62–4.71 (m, 2H, Oxd^{1}H5+Oxd^{2}H5), 5.00 (dd, *J* = 2.8, 8.8 Hz, 1H, Oxd^{2}H4), 5.99 (dd, *J* = 3.6, 9.6 Hz, 1H, Oxd^{1}H4), 7.07 (s, 1H, $CONH_2$), 7.35 (d, *J* = 8.1 Hz, 2H, ArH), 7.76 (s, 1H, $CONH_2$), 7.93 (d, *J* = 8.1 Hz, 2H, ArH); ^{13}C NMR (9 : 1 $CDCl_3$–DMSO-d_6) *δ* 21.1, 50.6, 57.4, 62.9, 63.2, 128.2, 129.7, 132.7, 137.9, 151.4, 154.1, 167.9, 170.6; RP-HPLC (see General methods) 5.7 min; ES-MS *m*/*z* 398.0 [M + 1], calcd 398.1; Elem. Anal. for $C_{15}H_{15}N_3O_8S$, calcd: C 45.34, H 3.80, N 10.57, S 8.07; found: C 45.28, H 3.82, N 10.60, S 8.10.

Ts-Oxd^1-Oxd^2-OMe (9). A stirred solution of Ts-Ser-Ser-OMe (**8**, 0.2 g, 0.56 mmol) in DCM (4 mL), was treated as described above for the synthesis of **7**. The same work up gave **9** (0.187 g, 81%, 94% pure by analytical RP-HPLC) and **10** (0.016 g, 8%), separated by flash chromatography.

(**9**). IR (nujol) *ν* 1779, 1769, 1721; ^{1}H NMR ($CDCl_3$) *δ* 2.45 (s, 3H, Me), 3.82 (s, 3H, COOMe), 4.39 (dd, *J* = 3.6, 9.6 Hz, 1H, Oxd^{1}H5), 4.50 (dd, *J* = 2.2, 9.1 Hz, 1H, Oxd^{2}H5), 4.74 (t, *J* = 9.4 Hz, 1H, Oxd^{1}H5), 4.76 (t, *J* = 9.3 Hz, 1H, Oxd^{2}H5), 5.08 (dd, *J* = 1.8, 9.1 Hz, 1H, Oxd^{2}H4), 6.00 (dd, *J* = 3.4, 9.6 Hz, 1H, Oxd^{1}H4), 7.36 (d, *J* = 8.0 Hz, 2H, ArH), 7.98 (d, *J* = 8.0 Hz, 2H, ArH); ^{13}C NMR (2 : 1 $CDCl_3$–DMSO-d_6) *δ* 21.0, 51.5, 52.1, 55.7, 62.1, 62.5, 128.9, 129.3, 133.0, 137.4, 151.3, 153.3, 165.1, 166.6; RP-HPLC (see General methods) 7.81 min; ES-MS *m*/*z* 413.0 [M + 1], calcd 413.1; Elem. Anal. for $C_{16}H_{16}N_2O_9S$, calcd: C 46.60, H 3.91, N 6.79, S 7.78; found: C 46.55, H 3.88, N 6.76, S 7.75.

Ts-Oxd-Dha-OMe (10). A stirred solution of **8** (0.20 g, 0.56 mmol) in DMF (3.0 mL) was treated as described above for the synthesis of **7**. The work up gave **10** (0.169 g, 82%, 94% pure by analytical RP-HPLC) and **9** (9.2 mg, 4%), isolated by flash chromatography.

(**10**). IR (nujol) *ν* 1768, 1721, 1715; ^{1}H NMR ($CDCl_3$) *δ* 2.42 (s, 3H, Me), 3.88 (s, 3H, COOMe), 4.45 (dd, *J* = 4.6, 9.0 Hz, 1H, OxdH5), 4.52 (t, *J* = 9.2 Hz, 1H, OxdH5), 4.99 (dd, *J* = 4.4, 9.2 Hz, 1H, OxdH4), 6.05 (s, 1H,═CH), 6.65 (s, 1H,═CH), 7.34 (d, *J* = 8.4 Hz, 2H, ArH), 7.93 (d, *J* = 8.4 Hz, 2H, ArH), 8.54 (s, 1H, DhaNH); ^{13}C NMR (2 : 1 $CDCl_3$–DMSO-d_6) *δ* 21.4, 52.6, 57.9, 66.0, 111.0, 128.7, 128.9, 129.1, 129.1, 131.0, 133.8, 145.4, 151.5, 163.6, 166.8; RP-HPLC (see General methods) 7.47 min; ES-MS *m*/*z* 369.1 [M + 1], calcd 369.1; Elem. Anal. for $C_{15}H_{16}N_2O_7S$, calcd: C 48.91, H 4.38, N 7.60, S 8.70; found: C 48.88, H 4.35, N 7.57, S 8.68.

Ts-Oxd^1-(5-Me-Oxd^2)-OMe (12a). A stirred solution of Ts-Ser-ThrOMe (**11a**, 0.20 g. 0.53 mmol) in DCM (4 mL), was treated as described above for the synthesis of **9**, giving **12a** (0.20 g 88%, 94% pure by analytical RP-HPLC). IR (nujol) *ν*, 1788, 1768, 1719; ^{1}H NMR ($CDCl_3$) *δ* 1.63 (d, *J* = 6.0 Hz, 3H, 5-Me), 2.46 (s, 3H, Ts*Me*), 3.82 (s, 3H, COOMe), 4.39 (dd, *J* = 3.2, 9.2 Hz, 1H, Oxd^{1}H5), 4.64 (d, *J* = 5.0 Hz, 1H, Oxd^{2}H4), 4.73 (br.t, 2H, Oxd^{1}H5 + Oxd^{2}H5), 6.04 (dd, *J* = 4.0, 10.0 Hz, 1H, Oxd^{1}H4), 7.36 (d, *J* = 8.0 Hz, 2H, ArH), 7.99 (d, *J* = 8.0 Hz, 2H, ArH); ^{13}C-NMR (9 : 1 $CDCl_3$–DMSO-d_6) *δ* 20.6, 21.3, 53.5, 57.6, 60.6, 65.0, 75.1, 128.9, 129.0, 133.7, 145.6, 151.2, 152.0, 167.1, 167.7; RP-HPLC (see General methods) 8.33 min; ES-MS *m*/*z* 427.3 [M + 1], calcd 427.1; Elem. Anal. for $C_{17}H_{18}N_2O_9S$, calcd: C 47.89, H 4.25, N 6.57, S 7.52; found: C 47.93, H 4.2 N 6.60, S 7.49.

Ts-D-Oxd^1-(5-Me-Oxd^2)-OMe (12b). The reaction of Ts-D-Ser-ThrOMe (**11b**, 0.20 g. 0.53 mmol) under the same conditions as described for **9** gave **12b** (0.196 g, 86%, 94% pure by analytical RP-HPLC). IR (nujol) *ν*, 1786, 1760, 1724; ^{1}H NMR ($CDCl_3$) *δ* 1.53 (d, *J* = 6.0 Hz, 3H, 5-Me), 2.45 (s, 3H, Ts*Me*), 3.90 (s, 3H, COOMe), 4.27 (dd, *J* = 3.2, 10.0 Hz, 1H, Oxd^{1}H5), 4.53 (d, *J* = 5.3 Hz, 1H, Oxd^{2}H4), 4.72 (t, *J* = 9.2 Hz, 1H, Oxd^{1}H4), 4.74 (t, *J* = 6.0 Hz, 1H, Oxd^{2}H5), 5.98 (dd, *J* = 3.2, 9.6 Hz, 1H, Oxd^{1}H4), 7.33 (d, *J* = 8.0 Hz, 2H, ArH), 7.96 (d, *J* = 8.0 Hz, 2H, ArH); ^{13}C NMR (2 : 1 $CDCl_3$–DMSO-d_6) *δ* 20.9, 21.7, 53.5, 58.2, 61.7, 65.2, 75.3, 129.3, 129.4, 134.2, 145.7, 151.3, 152.2, 167.0, 167.6; RP-HPLC (see General methods) 8.33 min; ES-MS *m*/*z* 427.3 [M + 1], calcd 427.1; Elem. Anal. for $C_{17}H_{18}N_2O_9S$, calcd: C 47.89, H 4.25, N 6.57, S 7.52; found: C 47.93, H 4.2 N 6.60, S 7.49.

Di-Oxd-peptide solid-phase synthesis

Ts-Oxd^1-Phe^2-Oxd^3-Phe^4-OH (14). Wang resin pre-loaded with Fmoc-Phe (0.5 g, 0.4–0.8 mmol g^{-1}, resin particle size: 100–200 mesh) was introduced in one reactor of an automated synthesizer apparatus.

Fmoc was removed with 4 : 1 DMF–piperidine (5 mL) under mechanical shaking. After 15 min, the suspension was filtered, the resin was washed with DCM (5 mL) and treated while shaking with a second portion of 4 : 1 DMF–piperidine. After 40 min, the suspension was filtered, and the resin was washed three times in sequence with DCM (5 mL) and CH_3OH (5 mL).

The resin was swollen in DCM (5 mL), and a solution of the *N*-protected amino acid (1.2 equiv.) and HOBt (1.2 equiv.) in DMF (4 mL) was added, followed by DCC (1.2 equiv.). The mixture was mechanically shaken, and after 3 h the resin was filtered and washed three times with the sequence DCM (5 mL), CH_3OH (5 mL). Coupling efficacy was determined by means of the Kaiser test.

The resin-bound peptide was suspended in 5 : 1 DCM–DMF (5 mL), and DSC (5 equiv.) and DIPEA (0.3 equiv.) were added at r.t. under inert atmosphere. After 3 h the mixture was filtered, and the resin-bound peptide was washed three times in sequence with DCM (5 mL) and CH_3OH (5 mL).

The resin-bound peptide was suspended in a mixture of TFA (1 mL), H_2O (0.33 mL), ethanedithiol (0.33 mL) and PhOH (0.33 mL) in DCM (8 mL), and mechanically shaken at r.t. After 2 h the mixture was filtered, the resin was washed twice with 5% TFA in Et_2O (5 mL), twice with Et_2O (5 mL). The cleavage

procedure was repeated, and all of the filtrates and washes were collected; solvent and volatiles were removed under N_2 flow at r. t. The resulting residue was suspended in Et_2O, and the crude solid which precipitated was triturated and collected by centrifuge. The Oxd-peptide acid **14** was isolated by semipreparative RP-HPLC (General methods) (80%, 96% pure by analytical RP-HPLC). IR (nujol) ν 3300–2900, 1784, 1776, 1727, 1719; 1715; ^{1}H NMR (9 : 1 $CDCl_3$–DMSO-d_6) δ 2.37 (s, 3H, Me), 2.70 (dd, J = 3.0, 14.0 Hz, 1H, Phe^{2}Hβ), 3.17 (dd, J = 6.0, 13.7 Hz, 1H, Phe^{4}Hβ), 3.27 (dd, J = 3.8, 14.0 Hz, 1H, Phe^{2}Hβ), 3.41 (dd, J = 5.8, 13.7 Hz, 1H, Phe^{4}Hβ), 3.98 (dd, J = 4.0, 8.4 Hz, 1H, Oxd^{3}H5), 4.25 (t, J = 9.0 Hz, 1H, Oxd^{3}H5), 4.45 (t, J = 8.9 Hz, 1H, Oxd^{1}H5), 4.52 (dd, J = 4.1, 8.3 Hz, 1H, Oxd^{1}H5), 4.64 (dd, J = 4.1, 7.9 Hz, 1H, Oxd^{3}H4), 4.79 (dd, J = 3.8, 8.4 Hz, 1H, Oxd^{1}H4), 4.83 (q, J = 6.6 Hz, 1H, Phe^{4}Hα), 5.78 (q, J = 6.8 Hz, 1H, Phe^{2}Hα), 6.54 (d, J = 7.6 Hz, 1H, Phe^{4}NH), 6.67 (d, J = 7.2 Hz, 1H, Phe^{2}NH), 7.15–7.30 (m, 12H, Phe^{2}ArH + Phe^{4}ArH + TsArH), 7.76 (d, J = 8.4 Hz, 2H, ArH); ^{13}C NMR (2 : 1 $CDCl_3$–DMSO-d_6) δ 23.9, 36.1, 37.8, 53.4, 54.1, 56.2, 59.2, 61.9, 62.8, 125.9, 127.7, 127.7, 128.3, 128.6, 128.8, 128.9, 129.3, 129.5, 133.7, 136.6, 136.6, 137.6, 151.9, 153.1, 171.7, 171.7, 174.7, 175.2; RP-HPLC (see General methods) 10.39 min; ES-MS m/z 693.2 [M + 1], calcd 693.2; Elem. Anal. for $C_{33}H_{32}N_4O_{11}S$, calcd: C 57.22, H 4.66, N 8.09, S, 4.63; found: C 57.18, H 4.69, N 8.12, S, 4.64.

Theoretical computations

All theoretical calculations were performed employing the HyperChem package.[47] The structures of the product **D**, and of the plausible reaction intermediates **A**, **C**, and **D**, were calculated employing *ab initio* molecular orbital (MO) theory. A systematic conformational analysis for the structures was done at the HF/6-31G* level. The conformers were re-optimized at the HF/6-31G** level. Optimization was performed by conjugate gradient algorithm, convergence at 0.001; energies are expressed in kcal mol^{-1}. The following molecules were included in the computations of **A–D**: **A**, DIPEA; **B**, DIPEAH$^+$; **C**, DIPEAH$^+$; **D**, 1-hydroxypyrrolidine-2,5-dione, and DIPEA.

Conformational analysis

Circular dichroism. ECD spectra were recorded from 200 to 300 nm at 25 °C. Solutions were made up in spectral grade solvents and run in a 0.01 cm quartz cell. For each sample the absorbance value was set to 1.0 at λ_{max} (225–260 nm); concentrations used were in the range 5–11 mM. Data are reported in ellipticity (millidegree).

NMR analyses. ^{1}H NMR spectra were recorded at 400 MHz in 5 mm tubes, using 0.01 M peptide at room temperature. Solvent suppression was performed by the solvent presaturation procedure implemented in Varian (PRESAT). ^{13}C NMR spectra were recorded at 75 MHz. Chemical shifts are reported as δ values. The unambiguous assignment of ^{1}H NMR resonances was performed by 2D gCOSY, HMBC, and HSQC. gCOSY experiments were conducted with a proton spectral width of 3103 Hz. VT-^{1}H-NMR experiments were performed over the range of 298–348 K. 2D spectra were recorded in the phase sensitive mode and processed using a 90°-shifted, squared sine-bell apodization. 2D ROESY experiments were recorded in the biomimetic medium 8 : 2 DMSO-d_6–H_2O, with a 250 ms mixing time with a proton spectral width of 3088 Hz. Peaks were calibrated on DMSO.

ROESY and molecular dynamics. Only ROESY-derived constraints were included in the restrained molecular dynamics. Cross-peak intensities were classified as very strong, strong, medium, and weak, and were associated with distances of 2.2, 2.6, 3.0, and 4.5 Å, respectively. Geminal couplings and other obvious correlations were discarded. For the absence of Hα(i, i + 1) ROESY cross peaks, all of the ω bonds were set at 180° (force constant: 16 kcal mol^{-1} Å^{-2}). The restrained MD simulations were conducted using the AMBER force field[48] in a 30 × 30 × 30 Å box of standard TIP3P models of equilibrated water.[49] All water molecules with atoms that come closer than 2.3 Å to a solute atom were eliminated. A 100 ps simulation at 1200 °C was used for generating 50 random structures that were subsequently subjected to 50 ps restrained MD with a 50% scaled force field at the same temperature, followed by 50 ps with full restraints (distance force constant of 7 kcal mol^{-1} Å^{-2}), after which the system was cooled in 20 ps to 50 °C. H-bond interactions were not included, nor were torsion angle restraints. The resulting structures were minimized with 3000 cycles of steepest descent and 3000 cycles of conjugated gradient (convergence of 0.01 kcal Å^{-1} mol^{-1}). The backbones of the structures were clustered by the rmsd analysis module of HyperChem.[44]

Unrestrained MD simulation was performed in a 30 × 30 × 30 Å box of standard TIP3P water for 10 ns at 298 °C, at constant temperature and pressure (Berendsen scheme,[50] bath relaxation constant of 0.2). For 1–4 scale factors, van der Waals and electrostatic interactions are scaled in AMBER to half their nominal value. The integration time step was set to 0.1 fs.

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