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## A convenient synthetic route to (2*S*,4*S*)-methylproline and its exploration for protein engineering of thioredoxin<sup>†</sup>

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**4-Substituted prolines, especially 4-fluoroprolines, have been widely used in protein engineering and design. Here, we report a robust and stereoselective approach for the synthesis of (2*S*,4*S*)-methylproline starting from (2*S*)-pyroglutamic acid. Incorporation studies with both (2*S*,4*R*)- and (2*S*,4*S*)-methylproline into the Trx1P variant of the model protein thioredoxin of *E. coli* show that the stereochemistry of the 4-methyl group might be a key determinant for successful incorporation during ribosomal synthesis of this protein.**

Proline (Pro) presents unique features among the proteinogenic amino acids and plays crucial structural and functional roles in peptides and proteins.<sup>1</sup> Two of its backbone atoms are constrained in the pyrrolidine ring, thus increasing the local structural rigidity. In peptides and proteins, the likelihood for the adoption of a *cis*-peptide bond increases from 0.04% for non-prolyl peptide bonds up to 6% for Xaa-Pro bonds (Xaa = any amino acid).<sup>2</sup> The *trans*-to-*cis*-isomerisation of prolyl-peptide bonds is a source of slow folding phases both *in vivo* and *in vitro* for proteins containing a *cis*-Pro in the native state.<sup>3</sup> The pyrrolidine ring of Pro adopts mainly the *C<sup>γ</sup>-endo* (*C<sup>γ</sup>* and Pro carbonyl group are on the same side of the plane) or the *C<sup>γ</sup>-exo* (*C<sup>γ</sup>* and Pro carbonyl group are on opposite sides of the plane) pucker. Both the conformational preference of the ring and the *cis/trans* equilibrium of the peptide bond can be modulated by installing electron-withdrawing or sterically demanding substituents on the *C<sup>γ</sup>* atom.<sup>4</sup> In fact, the *endo* pucker conformation enhances the stability of the *cis* peptide bond, while the *exo* conformation enhances the stability of the *trans* peptide bond.<sup>5</sup> Therefore, 4-substituted Pro analogues (especially 4-fluoroproline analogues) have been widely used in molecular design *e.g.* for tuning the conformational stability of peptides and proteins,<sup>6</sup> for investigations of protein folding

kinetics,<sup>7</sup> for studying structure–activity relationships,<sup>8</sup> and for developing novel peptide-based materials.<sup>9</sup> However, 4-methylprolines (4-Mep), that could explore steric instead of electronic effects, have been by far less exploited in peptide/protein engineering in comparison to 4-fluoroproline analogues. A convenient access to 4-Mep could enable such complementary studies to those performed with 4-fluoroproline.

Furthermore, the non-proteinogenic amino acid (2*S*,4*S*)-methylproline (4*S*-Mep) is present in several secondary metabolites, predominantly from marine organisms, endowed with antibiotic, anticancer, cytotoxic, and anti-proteolytic activities.<sup>10</sup> Therefore, a simple and highly diastereoselective synthetic route for 4*S*-Mep would also be advantageous for the synthesis of various natural products and drugs. The 4-Mep commercially available diastereomers, especially (2*S*,4*S*)-Mep, are more expensive than the corresponding 4-fluoroprolines analogues. Although approaches for the synthesis of 4-Mep are reported in the literature, most protocols do not offer a unique synthetic route to each diastereomer. Moreover, difficult separations, long synthetic routes and the use of unusual and/or expensive reagents are required in order to obtain both 4-Mep isomers with high enantioselectivity.<sup>11</sup> The original protocol for the synthesis of 4-Mep reported by Del Valle and Goodman relied on stereodivergent hydrogenations of a 4-exomethylene-proline.<sup>11a</sup> A more recent procedure involving the use of Crabtree's catalyst provided a succinct synthesis of 4-methylprolines with an improved stereoselectivity for the 4*R*-Mep derivative.<sup>11b</sup> An alternative approach to catalytic hydrogenation based on the use of sterically bulky Evans' chiral auxiliaries was also recently reported for the synthesis of the four diastereoisomers of Boc-protected 4-methylproline.<sup>11c</sup> Finally, a novel synthetic route that enables the control of the stereochemistry of the substituents at the *C<sup>γ</sup>* position by employing phase transfer catalysis was later reported by Hulme and co-workers.<sup>11g</sup>

Most synthetic routes towards the synthesis of 4-Mep involve the hydrogenation of a 4-methylenepyrrolydine intermediate, sometimes entailing expensive and toxic catalysts (Table 1).

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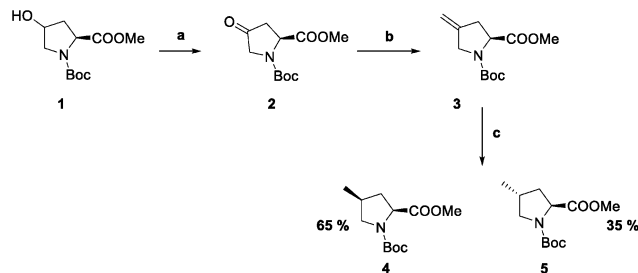
**Table 1** Hydrogenation conditions for 4-methylenepyrrolydine intermediates during 4-Mep synthesis

Substrate	Reaction conditions	dr <sup>a</sup>	Ref.
	Crabtree's catalyst, CHCl <sub>3</sub> , H <sub>2</sub> , rt, 5 days	4S : 4R 1 : 15	11b
	Pd/C, CH <sub>2</sub> Cl <sub>2</sub> , H <sub>2</sub> , rt, 16 h	4S : 4R 70 : 10	11b
	PtO <sub>2</sub> , MeOH, 1 atm, 20 h	4S : 4R 80 : 20	12
	Pd/C 5%, H <sub>2</sub> O, 2.96 atm, 8 h	4S : 4R 90 : 10	13
	Pd/C 10%, 2-propanol, 1 atm, 18 h	4S : 4R 16 : 1	14
	PtO <sub>2</sub> , MeOH, 1 atm, 12 h	4S : 4R 50 : 10	15
	Crabtree's catalyst, CH <sub>2</sub> Cl <sub>2</sub> , H <sub>2</sub> , 1 atm, 18 h	4S : 4R 1 : 40	11a
	(1) Pd/C, MeOH, 1 bar	(1) 4S : 4R 25 : 10	11a
	(2) RANEY®-Ni, MeOH, 1 bar	(2) 4S : 4R 30 : 10	11a

<sup>a</sup> Diastereomeric ratio.

All reported procedures strive to increase the poor control of the stereochemistry of the 4-methyl group during hydrogenation using sterically hindered catalysts or substituents on the modified proline. In this work, we report on a different strategy that yields a robust and highly stereoselective approach for the synthesis of 4S-Mep, based on cheap and commercially available (2S)-pyroglutamic acid.

Initially, we reproduced the hydrogenation protocol reported by Munro and coworkers using (2S,4R/S)-hydroxyproline as starting material (Scheme 1). Briefly, the carboxylic group was protected by esterification with thionyl chloride in methanol followed by *N*-*tert*-butyl-oxycarbonylation (Boc protection) using published protocols.<sup>16</sup> Compound 1 was oxidized with trichloroisocyanuric acid (TCCA) and catalytic TEMPO to afford 2 in 90% yield after flash chromatography purification.<sup>11a</sup> Wittig olefination of the resulting oxopyrrolidine was undertaken according to literature procedures to deliver 3 with a 35% overall yield after purification.<sup>17</sup> Hydrogenation of compound 3 with 10% Pd/C gave isomers 4 and 5 (ratio 4S : 4R 65 : 35), consistent with literature data. The ratio between the two diastereoisomers was determined using a chiral HPLC column packed with cellulose tris (4-methylbenzoate) coated on silica-gel. NMR analysis on

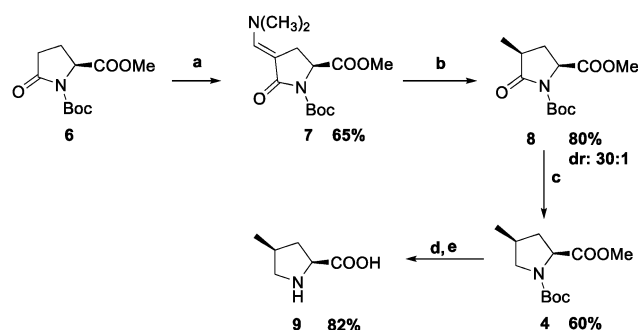
**Scheme 1** Synthetic route to (2S,4S)-methylproline and (2S,4R)-methylproline starting from protected (2S,4R/S)-hydroxyproline: (a) TCCA, TEMPO, CH<sub>2</sub>Cl<sub>2</sub>; (b) KOtBu, CH<sub>3</sub>P(Ph)<sub>3</sub>Br, THF; (c) EtOAc, Pd/C.

the  $\alpha$  peak areas confirmed the ratio calculated using analytical HPLC (see ESI†). The stereoselectivity for the synthesis of (4S)-Mep achieved in this route was not satisfactory for our purpose of incorporating this non-proteinogenic amino acid into a model protein. However, this route can be exploited for the synthesis of further modified proline analogues. In fact, we used intermediate 2 to synthesize 4,4-difluoroproline in good yields using DAST (diethylaminosulfur trifluoride) for nucleophilic fluorination.<sup>18</sup>

We then established an alternative synthetic route to (4S)-Mep to enhance stereoselectivity during hydrogenation using the Pd/C catalyst (Scheme 2). (2S)-Pyroglutamic acid was subjected to esterification with thionyl chloride in methanol followed by protection of the amino group with *tert*-butyloxycarbonyl (Boc) according to literature procedures to give compound 6.<sup>16b</sup>

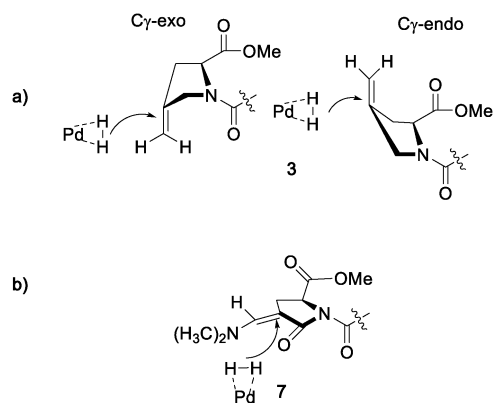
Compound 6 was reacted with Bredereck's reagent in toluene at 100 °C to give the enaminone 7 in 65% yield.

Bredereck's reagent is used for this step as it has been reported that direct methylation of pyroglutamate ester analogs using methyl iodide in the presence of a strong base leads to a mixture of monoalkylated and dialkylated products without *cis*-stereospecificity.<sup>19</sup> The enaminone 7 was hydrogenated with Pd/C (200 psi) in isopropanol/ethyl acetate to give the 4S-methylpyroglutamate 8 as the main product by a

**Scheme 2** Synthetic route to (2S,4S)-methylproline starting from (2S)-pyroglutamic acid: (a) Bredereck's reagent, toluene, 100 °C; (b) 10% Pd/C, iPrOH/EtOAc, 200 psi (c) BH<sub>3</sub>Me<sub>2</sub>S, THF, 40 °C; (d) 1M LiOH, THF; (e) CH<sub>2</sub>Cl<sub>2</sub>/TFA.

process involving reduction, elimination and reduction. In fact, the less hindered  $C^{\gamma}$ -endo side was almost quantitatively preferred during the hydrogenation with Pd/C resulting in high *cis*-stereospecificity (dr = 30 : 1), which is consistent with literature reports for similar reactions<sup>16b,20</sup> and represents the highest selectivity for the installation of the 4*S*-methyl group into the proline scaffold reported to date. The carbonyl group on  $C^{\delta}$  was reduced chemoselectively using borane dimethylsulfide (BH<sub>3</sub>Me<sub>2</sub>S) to give compound 4. Using NMR and mass spectroscopy analysis, we identified the Boc protected (2*S*,4*S*)-methylprolinol as the main by-product of this reduction reaction, which became the major product when the reaction time was increased above 16 h or a large excess (>2.5 eq.) of BH<sub>3</sub>Me<sub>2</sub>S was used. Methylprolinol can be recovered and the hydroxyl group can be oxidized with Jones reagent to minimise loss of material.<sup>11a,21</sup> The following steps were the hydrolysis of the methyl ester using LiOH and finally the cleavage of the Boc protecting group under mild conditions (TFA/CH<sub>2</sub>Cl<sub>2</sub>) to give the (2*S*,4*S*)-Mep 9.<sup>22</sup>

All procedures reported so far for the hydrogenation of a methylene pyrrolidine substrate showed that the crucial step for stereoselectivity is the heterogeneous hydrogenation.<sup>10b,11a,b,15,16b,20d</sup> During the hydrogenation catalyzed by Pd/C, the alkene is absorbed on the surface of the catalyst before the hydrogen shifts from the metal to the carbon sp<sup>2</sup> orbitals. The resulting saturated hydrocarbon leaves the catalyst surface. During this process, the preferential face of hydrogenation of the substrate depends on the puckering of the molecule. If no steric constraint is present, the hydrogenation results in a low stereoselectivity because of the presence of different puckering isomers.<sup>23</sup> The predominant ring pucker of substituted proline can be biased by hydrogen bonding or by the functionalization of  $C^{\gamma}$  with either hindered functional groups or electronegative substituents.<sup>4</sup> Hydrogenation of 3 (Scheme 3a) with Pd/C showed a slight preference for the formation of the 4*S* product. This preference can be explained by the favouring of its *endo* ring pucker in solution. H<sub>2</sub> will be added across the C–C double bond at the less hindered face, resulting in a preference for the 4*S* product when 3 adopts the *endo* pucker. Nevertheless, a



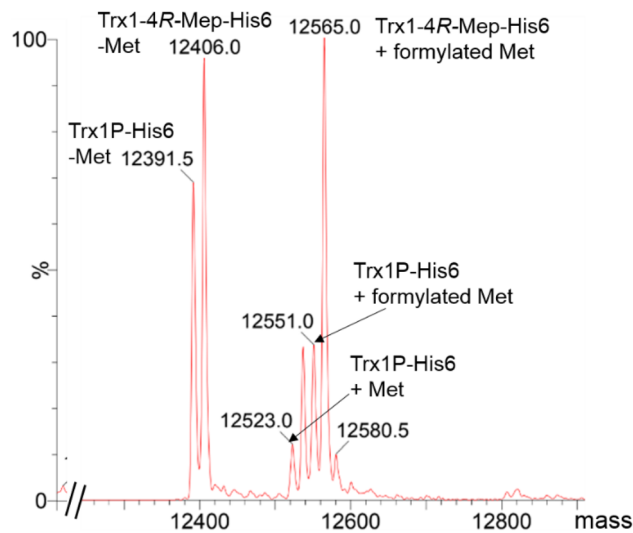
**Scheme 3** Hydrogen addition preferences for both ring faces for compounds 3 and 7.

significant amount of the 4*R* product is also formed as 3 also significantly populates the *exo* pucker. In contrast, the Boc-protected oxopyrrolidine ester 7 used in our synthesis adopts a single, pseudo planar conformation (Scheme 3b) due to the presence of the carboxy group on the  $C^{\delta}$  carbon. For this reason, the hydrogenation of 7 occurred almost exclusively on the less hindered face of the pyrrolidone ring, resulting in the formation of the 4*S* diastereomer. In this case, the stereoselectivity is elicited by the steric bulk of the substituents around the double bond, as previously observed.<sup>16b</sup>

Next, we investigated the suitability of both 4-Mep isomers as substrates for ribosomal translation. We attempted the incorporation of 4*S*-Mep and commercially available 4*R*-Mep into the thioredoxin variant Trx1P of *E. coli*, which contains *cis*Pro76 as a single proline residue.<sup>6c</sup>

Briefly, expression of Trx1P with a His6 tag at the C-terminus (Trx1P-His6) in the presence of 2.5 mM 4*S*- or 4*R*-Mep was performed in M9 minimal medium as previously described,<sup>7b</sup> using the proline auxotrophic cell strain CAG18515 transformed with an additional plasmid for over-expression of an engineered *E. coli* prolyl-tRNA-synthetase, ProRS (C443G) to increase the incorporation of the non-natural proline analogue.<sup>24</sup> After purification by Ni-NTA affinity chromatography, the identity of the purified protein was assessed by mass spectrometry.

In the presence of 4*S*-Mep only wild type Trx1P was detected. In-gel trypsin digest was performed to exclude the



**Fig. 1** Deconvoluted ESI-MS spectrum of *E. coli* Thioredoxin variant Trx1P-His<sub>6</sub> produced in M9 minimal medium in the presence of 4*R*-Mep by the proline auxotrophic bacterial strain CAG 18515. The found molecular weight of the parent protein missing the initial methionine was 12 391.5 Da. The peak corresponding to 12 406 Da represents the modified protein with the incorporated 4*R*-Mep at position 76 (Trx1-4*R*-Mep-His6). Further peaks correspond to the parent protein with the initial methionine (12 523 Da; Trx1P-His6 + Met), the parent protein with formylated initial methionine (12 551 Da; Trx1P-His6 + formylated Met), and to the modified protein with the incorporated 4*R*-Mep at position 76 with initial formylated methionine (Trx1-4*R*-Mep-His6 + formylated Met).



accumulation of the modified protein in inclusion bodies due to solubility issues. However, the peptide fragments also only showed natural Pro at position 76, indicating that either 4*S*-Mep had not been incorporated into the protein (e.g. due to poor recognition by *E. coli* prolyl-tRNA-synthetase) or that the protein containing 4*S*-Mep at position 76 was intracellularly degraded due to destabilization or misfolding. Conversely, 4*R*-Mep could be incorporated into Trx1P under the same conditions in place of *cis*Pro76 with more than 60% incorporation yield (Fig. 1). These results could also potentially be explained by the pucker preferences of the 4-Mep isomers. In fact, 4*S*-Mep favours the *exo* pucker of the pyrrolydine ring, while 4*R*-Mep favours the adoption of an *endo* pucker.<sup>6i</sup> In the thioredoxin fold there is an almost unique preference for the *endo* pucker of the conserved Pro, which is in *cis* conformation, in the context of the tertiary structure.<sup>6c</sup> Therefore, the partial incorporation of 4*R*-Mep, could potentially be attributed to the preferred adoption of the *endo* pucker in the protein's tertiary structure. In fact, we have previously reported that the pucker preferences of proline analogues can play a substantial role in the success of incorporation experiments.<sup>7c</sup>

## Conclusions

(2*S*,4*S*)-4-Methylproline was successfully synthesized in 30 : 1 dr from (2*S*)-pyroglutamic acid as the starting material. This synthetic route is based on the use of reagents and equipment readily available also in resource-limited settings. We have reported for the first time the attempt to incorporate 4-Mep isomers into a globular protein. The results from the incorporation experiments with both 4-Mep isomers into the model protein Trx1P showed the suitability of (4*R*)-Mep for ribosomal protein translation. Further studies will be needed to fully explore the reasons behind the lack of incorporation of (4*S*)-Mep into this protein. These and similar studies will be enabled by the straightforward synthesis method established here.

## Author contributions

Andrea Caporale: investigation, data curation, methodology, conceptualization, writing first draft. Jennie O'Loughlin: methodology, investigation, writing first draft. Yannick Ortin: NMR data analysis. Marina Rubini: conceptualization, supervision, funding acquisition, finalisation of manuscript.

## Conflicts of interest

There are no conflicts to declare.

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## Notes and references

- (a) M. D. Shoulders and R. T. Raines, *Annu. Rev. Biochem.*, 2009, **78**, 929–958; (b) K. P. Lu, G. Finn, T. H. Lee and L. K. Nicholson, *Nat. Chem. Biol.*, 2007, **3**, 619–629; (c) G. Fischer, *Chem. Soc. Rev.*, 2000, **29**, 119–127.
- A. Jabs, M. S. Weiss and R. Hilgenfeld, *J. Mol. Biol.*, 1999, **286**, 291–304.
- U. Reimer, G. Scherer, M. Drewello, S. Kruber, M. Schutkowski and G. Fischer, *J. Mol. Biol.*, 1998, **279**, 449–460.
- H. K. Ganguly and G. Basu, *Biophys. Rev.*, 2020, **12**, 25–39.
- (a) M. L. DeRider, S. J. Wilkens, M. J. Waddell, L. E. Bretscher, F. Weinhold, R. T. Raines and J. L. Markley, *J. Am. Chem. Soc.*, 2002, **124**, 2497–2505; (b) N. V. Costantini, H. K. Ganguly, M. I. Martin, N. A. Wenzell, G. P. A. Yap and N. J. Zondlo, *Chem. – Eur. J.*, 2019, **25**, 11356–11364.
- (a) T. Steiner, P. Hess, J. H. Bae, B. Wiltschi, L. Moroder and N. Budisa, *PLoS One*, 2008, **3**, e1680; (b) C. Renner, S. Alefelder, J. H. Bae, N. Budisa, R. Huber and L. Moroder, *Angew. Chem., Int. Ed. Engl.*, 2001, **40**, 923–925; (c) M. Rubini, M. A. Scharer, G. Capitani and R. Glockshuber, *ChemBioChem*, 2013, **14**, 1053–1057; (d) D. Naduthambi and N. J. Zondlo, *J. Am. Chem. Soc.*, 2006, **128**, 12430–12431; (e) T. Y. Zheng, Y. J. Lin and J. C. Horng, *Biochemistry*, 2010, **49**, 4255–4263; (f) K. Deepankumar, S. P. Nadarajan, N. Ayyadurai and H. Yun, *Biochem. Biophys. Res. Commun.*, 2013, **440**, 509–514; (g) U. Arnold and R. T. Raines, *Org. Biomol. Chem.*, 2016, **14**, 6780–6785; (h) H. C. Tang, Y. J. Lin and J. C. Horng, *Proteins*, 2014, **82**, 67–76; (i) M. D. Shoulders, J. A. Hodges and R. T. Raines, *J. Am. Chem. Soc.*, 2006, **128**, 8112–8113; (j) M. D. Shoulders, K. A. Satyshur, K. T. Forest and R. T. Raines, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 559–564; (k) S. K. Holmgren, L. E. Bretscher, K. M. Taylor and R. T. Raines, *Chem. Biol.*, 1999, **6**, 63–70; (l) M. D. Shoulders and R. T. Raines, *Adv. Exp. Med. Biol.*, 2009, **611**, 251–252; (m) D. Dietz, V. Kubyskin and N. Budisa, *ChemBioChem*, 2015, **16**, 403–406.
- (a) D. Roderer, R. Glockshuber and M. Rubini, *ChemBioChem*, 2015, **16**, 2162–2166; (b) J. O' Loughlin, S. Napolitano and M. Rubini, *ChemBioChem*, 2021, **22**, 3326–3332; (c) M. D. Crespo and M. Rubini, *PLoS One*, 2011, **6**(5), e19425.
- C. Held, H. Hubner, R. Kling, Y. A. Nagel, H. Wennemers and P. Gmeiner, *ChemMedChem*, 2013, **8**, 772–778.



- 9 (a) R. S. Erdmann and H. Wennemers, *J. Am. Chem. Soc.*, 2012, **134**, 17117–17124; (b) M. R. Aronoff, J. Egli, M. Menichelli and H. Wennemers, *Angew. Chem., Int. Ed.*, 2019, **58**, 3143–3146; (c) J. Egli, C. Siebler, B. Maryasin, R. S. Erdmann, C. Bergande, C. Ochsenfeld and H. Wennemers, *Chem. – Eur. J.*, 2017, **23**, 7938–7944; (d) R. S. Erdmann and H. Wennemers, *Angew. Chem., Int. Ed.*, 2011, **50**, 6835–6838.
- 10 (a) L. Liu, J. Jokela, L. Herfindal, M. Wahlsten, J. Sinkkonen, P. Permi, D. P. Fewer, S. O. Doskeland and K. Sivonen, *ACS Chem. Biol.*, 2014, **9**, 2646–2655; (b) K. Fukuhara, K. Takada, S. Okada and S. Matsunaga, *J. Nat. Prod.*, 2016, **79**, 1694–1697; (c) J. C. Kehr, D. G. Picchi and E. Dittmann, *Beilstein J. Org. Chem.*, 2011, **7**, 1622–1635; (d) T. Teruya, H. Sasaki, H. Fukazawa and K. Suenaga, *Org. Lett.*, 2009, **11**, 5062–5065.
- 11 (a) J. R. Del Valle and M. Goodman, *J. Org. Chem.*, 2003, **68**, 3923–3931; (b) A. C. Murphy, M. I. Mitova, J. W. Blunt and M. H. Munro, *J. Nat. Prod.*, 2008, **71**, 806–809; (c) K. Sun, C. Tao, B. Long, X. Zeng, Z. Wu and R. Zhang, *RSC Adv.*, 2019, **9**, 32017–32020; (d) A. M. P. Koskinen and H. Rapoport, *J. Org. Chem.*, 1989, **54**, 1859–1866; (e) C. Heindl, H. Hübner and P. Gmeiner, *Tetrahedron: Asymmetry*, 2003, **14**, 3153–3172; (f) S. R. Crosby, R. B. Sessions and C. L. Willis, *Synlett*, 2010, 539–542; (g) H. J. Johnston, F. S. McWhinnie, F. Landi and A. N. Hulme, *Org. Lett.*, 2014, **16**, 4778–4781.
- 12 S. K. Das, Y. L. Bennani, B. Liu, C. Cadilhac, J. Henderson, R. Denis, C. Poisson, M. A. Morris, L. C. C. Kong, T. J. Reddy, C. Yannopoulos, S. Giroux, L. Vaillancourt, O. Z. Pereira, G. Falardeau and J. Maxwell, WO2011/119853, Vertex Pharmaceuticals Incorporated, 2011.
- 13 J. A. Henderson, J. Maxwell, L. Vaillancourt, M. A. Morris, R. Grey Jr., S. Giroux, L. C. C. Kong, S. K. Das, B. Liu, C. Poisson, C. Cadilhac, M. Bubenik, T. J. Reddy, G. Falardeau, C. Yannopoulos, J. Wang, O. Z. Pereira, Y. L. Bennani, A. C. Pierce, G. R. Bhisetti, K. M. Cottrell and V. Marone, WO2011/009084A2, Vertex Pharmaceuticals Incorporated, 2011.
- 14 R. Lavoie, J. A. Bender, Z. Yang, M. Belema, O. D. Lopez, Q. Chen, G. Wang and P. Hewawasam, WO2011/059887A1, Bristol-Myers Squibb, 2011.
- 15 C. Gardelli, E. Nizi, E. Muraglia, B. Crescenzi, M. Ferrara, F. Orvieto, P. Pace, G. Pescatore, M. Poma, R. Ferreira Mdel, R. Scarpelli, C. F. Homnick, N. Ikemoto, A. Alfieri, M. Verdirame, F. Bonelli, O. G. Paz, M. Taliani, E. Monteagudo, S. Pesci, R. Laufer, P. Felock, K. A. Stillmock, D. Hazuda, M. Rowley and V. Summa, *J. Med. Chem.*, 2007, **50**, 4953–4975.
- 16 (a) S. M. Dixon, K. A. Milinkevich, J. Fujii, R. Liu, N. Yao, K. S. Lam and M. J. Kurth, *J. Comb. Chem.*, 2007, **9**, 143–157; (b) E. Coudert, F. Acher and R. Azerad, *Synthesis*, 1997, **1997**(8), 863–865.
- 17 S. Loosli, C. Foletti, M. Pappmeyer and H. Wennemers, *Synlett*, 2019, **30**, 508–510. As previously reported in the literature, we could confirm that the reaction yields for the Wittig olefination could be increased from 35% to 70% by replacing the methylester with a *tert*-butyl ester.
- 18 J. R. Sufrin, T. M. Balasubramanian, C. M. Vora and G. R. Marshall, *Int. J. Pept. Protein Res.*, 1982, **20**, 438–442.
- 19 J. D. Charrier, J. E. S. Duffy, P. B. Hitchcock and D. W. Young, *J. Chem. Soc., Perkin Trans. 1*, 2001, 2367–2371.
- 20 (a) R. A. August, J. A. Khan, C. M. Moody and D. W. Young, *Tetrahedron Lett.*, 1992, **33**, 4617–4620; (b) C. M. Moody and D. W. Young, *Tetrahedron Lett.*, 1993, **34**, 4667–4670; (c) C. M. Moody and D. W. Young, *Tetrahedron Lett.*, 1994, **35**, 7277–7280; (d) M. Katoh, H. Mizutani and T. Honda, *Heterocycles*, 2006, **69**, 193–216.
- 21 U. K. Mishra and N. G. Ramesh, *Tetrahedron Lett.*, 2020, **61**, 152081.
- 22 M. Rodriguez, S. Terracciano, E. Cini, G. Settembrini, I. Bruno, G. Bifulco, M. Taddei and G.-P. Luigi, *Angew. Chem., Int. Ed.*, 2006, **45**, 423–427.
- 23 L. Massaro, J. Zheng, C. Margarita and P. G. Andersson, *Chem. Soc. Rev.*, 2020, **49**, 2504–2522.
- 24 W. Kim, A. George, M. Evans and V. P. Conticello, *ChemBioChem*, 2004, **5**, 928–936.

