Genetically encoded amino acids with *tert*-butyl and trimethylsilyl groups for site-selective studies of proteins by NMR spectroscopy

Choy Theng Loh^{1,2}, Luke A. Adams³, Bim Graham³, Gottfried Otting¹

¹ Research School of Chemistry, Australian National University, Canberra, ACT 2601 Australia

² Zhejiang Provincial Key Laboratory of Chemical and Biological Processing Technology of Farm Products, Zhejiang University of Science and Technology, Hangzhou 310023, PR China

³ Medicinal Chemistry, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville VIC 3052, Australia

Abstract

The amino acids 4-(*tert*-butyl)phenylalanine (Tbf) and 4-(trimethylsilyl)phenylalanine (TMSf), as well as a partially deuterated version of Tbf (dTbf), were chemically synthesized and site-specifically incorporated into different proteins, using an amber stop codon, suppressor tRNA and the broadband aminoacyl-tRNA synthetase originally evolved for the incorporation of *p*-cyano-phenylalanine. The ¹H-NMR signals of the *tert*-butyl and TMS groups were compared to the ¹H-NMR signal of *tert*-butyltyrosine (Tby) in protein systems with molecular weights ranging from 8 to 54 kDa. The ¹H-NMR resonance of the TMS group appeared near 0 ppm in a spectral region with few protein resonances, facilitating the observation of signal changes in response to ligand binding. In all proteins, the *R*₂ relaxation rate of the *tert*-butyl group of Tbf was only little greater than that of Tby (less than two-fold). Deuteration of the phenyl ring of Tbf made only a relatively small difference. The effective *T*₂ relaxation time of the TMS signal was longer than 140 ms even in the 54 kDa system.

Introduction

tert-Butyltyrosine (Tby, Fig. 1) is an unnatural amino acid that can be site-specifically incorporated into proteins in response to an amber stop codon. The *tert*-butyl group in *tert*-butyltyrosine appears in the 1D ^1H -NMR spectrum as an intense singlet. For solvent-exposed *tert*-butyl groups, this signal is also narrower than other resonances of the protein, due to its enhanced mobility arising from rapid rotations of the methyl groups and around the carbon–oxygen bonds linking the *tert*-butyl group to the aromatic ring of the amino acid. The narrow and intense singlet can thus readily be identified in the 1D ^1H -NMR spectrum even for protein systems of high molecular weight (> 300 kDa), producing a highly sensitive NMR probe for measuring submicromolar ligand binding affinities¹ and measuring intermolecular NOEs². The intensity of a ^1H -NMR signal produced by nine ^1H spins is a clear advantage over ^{19}F -NMR of unnatural amino acids containing a CF_3 group^{1,3,4}, especially as ^{19}F -NMR resonances are much more susceptible to broadening by chemical shift anisotropy than the ^1H -NMR signals of CH_3 groups.

The chemical shift of a *tert*-butyl group is typically at about 1.3 ppm in aqueous solution, with small variations depending on the local chemical environment. This means that, despite its high intensity and narrow lineshape, the ^1H -NMR resonance of the *tert*-butyl group can be masked by the protein background. In principle, this problem can be addressed by using Tby with a uniformly ^{13}C -labelled *tert*-butyl group, allowing its selective detection by a simple ^{13}C -HSQC spectrum. We recently demonstrated this approach with a 95 kDa protein-DNA complex², but it is expensive and therefore requires a highly efficient way of incorporation into proteins, such as cell-free protein synthesis⁴. A simpler approach would be to substitute Tby by an amino acid with a trimethylsilyl (TMS) group instead of the *tert*-butyl group. As the TMS group produces a singlet at about 0 ppm, it is in a spectral region where few protein resonances are found. We recently confirmed that a TMS tag ligated to the thiol group of a solvent-exposed cysteine produces a ^1H -NMR signal at 0 ppm that is readily observable even in systems of high molecular weight². In addition, it would be attractive if the location of the *tert*-butyl or TMS group could be predicted more accurately relative to the protein backbone by the design of a more rigid tether than in Tby.

Here we report the chemical synthesis of 4-(*tert*-butyl)phenylalanine-(phenyl-3,5- d_2) (dTbf), 4-(trimethylsilyl)phenylalanine (TMSf) and 4-((trimethylsilyl)methoxy)phenylalanine (TMSmy) for genetically encoded incorporation into proteins. Tbf, which is commercially available, dTbf and TMSf were successfully incorporated into different proteins using the polysubstrate-specific *p*-cyanophenylalanine aminoacyl-tRNA synthetase (*p*CNF-RS) reported by Peter G. Schultz and co-workers⁵, whereas the same system failed to incorporate TMSmy.

To the best of our knowledge, the present work is the first describing the successful incorporation of Tbf and TMSf into proteins. For comparison, we also incorporated Tby. As the *tert*-butyl and TMS groups contain nine equivalent protons generating a singlet in the ^1H -NMR spectrum, we refer to them as 9PS groups (nine proton singlet). To assess their relative merits, we investigated the $R_2(^1\text{H})$ relaxation rates of the 9PS amino acids in proteins of different molecular weight and tested TMSf as a probe for ligand binding.

Materials and methods

Materials

RF1-depleted S30 extract was prepared from the genomically modified *E. coli* strain BL21 Star (DE3)::RF1-CBD3 as described previously^{4,6}. Tby and Tbf were purchased from Sigma-Aldrich and Santa Cruz Biotechnology, respectively. Detailed protocols for the synthesis of TMSmy, TMSf and deuterated Tbf are provided in the supporting information. Total tRNA including optimized suppressor-tRNA was prepared as described previously⁷. *p*CNF-RS was expressed from the pEvol-*p*CNF plasmid⁵. The protease inhibitor 4-nitrophenyl-4-guanidinobenzoate hydrochloride was purchased from Sigma-Aldrich.

Expression and purification of *p*CNF-RS

The gene encoding *p*CNF-RS was amplified by PCR from pEvol-*p*CNF^{5,8}, cloned into the T7 vector pETMCSI⁹ and transformed into *E. coli* BL21(DE3). The cells were grown in 1 L of LB medium containing 100 $\mu\text{g/mL}$ ampicillin at 37 °C and induced with 1 mM IPTG at $\text{OD}_{600} = 0.7$. Cells were harvested after overnight expression (about 16 h) at 25 °C by centrifugation. Pellets were suspended in buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT and 10% glycerol) and cells were lysed by French press at 12,000 psi. The cell lysates were centrifuged for 1 h at 30,000 *g* at 4 °C. The supernatant was subsequently loaded onto a DEAE Toyopearl 650 M anion exchange column. *p*CNF-RS was eluted with a linear gradient of 0 – 600 mM NaCl in buffer A. Fractions containing *p*CNF-RS were pooled and dialyzed against buffer B (20 mM Tris-HCl, pH 8, 1 mM EDTA and 1 mM DTT). The concentrated solution of *p*CNF-RS was stored at -70 °C.

Preparation of total tRNA including optimized suppressor tRNA

The pEvol plasmid encoding optimized suppressor-tRNA⁸ was transformed into BL21(DE3) cells. Total tRNA containing optimized suppressor-tRNA was prepared as described⁷. About

20 mg of purified total tRNA was obtained from 8 L of LB medium. The concentration of total tRNA was measured by its absorbance at 260 nm.

Cell-free protein synthesis

Amber stop mutants of human ubiquitin (T66U), *S. aureus* sortase A (SrtA Y17U), rat ERp29 (S114U) and Zika virus NS2B-NS3 protease (Zipro *S81U, where the star indicates that the unnatural amino acid is in NS2B) were designed with a C-terminal His₆-tag and cloned into pETMCSI⁹. The proteins were expressed by continuous exchange cell-free protein synthesis. The cell-free reactions were carried out at 30 °C for 16 h as described previously^{4,6,7}. Additional components added into the cell-free reaction mixtures were: 0.2 mg/ml total tRNA including optimized suppressor-tRNA, 30 µM pCNF-RS in the inner reaction mixture and 1 mM unnatural amino acid in the inner and outer compartments. The volumes of the inner and outer mixtures were 2 and 20 mL, respectively. Proteins were purified using a 1 mL Ni-NTA gravity column equilibrated with buffer C (50 mM Tris-HCl, pH 8.0, 300 mM NaCl) and washed with buffer D (same as buffer C but with 20 mM imidazole). Proteins were then eluted with buffer E (same as buffer C but with 300 mM imidazole). Purified ubiquitin was buffer-exchanged and concentrated against NMR buffer F (50 mM HEPES-KOH, pH 7.0) by ultrafiltration using an Amicon centrifugal filter unit. The other proteins were buffer-exchanged and concentrated against NMR buffer G (20 mM MES-KOH, pH 6.5, 20 mM NaCl) in the same way. Filter cutoffs were 3 kDa for ubiquitin and 10 kDa for the other proteins.

Tby mutant of *Mt* LeuRS by *in vivo* protein expression

The gene encoding the Y115U mutant of C-terminal His₆-tagged *M. thermoautotrophicum* leucyl-tRNA synthetase (LeuRS Y115U) was cloned into pETMCSI⁹ and co-transformed with pEvol-pCNF⁵ into *E. coli* B-95.ΔA¹⁰. Cells were grown in 20 mL of LB medium containing 100 µg/mL ampicillin, 33 µg/mL chloramphenicol and 0.02% arabinose, induced with 1 mM IPTG at OD₆₀₀ = 0.5 and supplemented with 1 mM Tby. The cells were harvested after overnight expression by centrifugation at 25 °C. The pellet was re-suspended in buffer C, lysed by ultra-sonication and the lysate was centrifuged for 1 h at 30,000 g at 4 °C. The supernatant was loaded onto a 1 mL Ni-NTA gravity column and the purification was carried out as described above. Purified protein was buffer-exchanged and concentrated against NMR buffer G by ultrafiltration.

NMR and *R*₂ relaxation measurement

NMR spectra were recorded at 25 °C on a 800 MHz Bruker NMR spectrometer equipped with a cryoprobe, using the double spin echo sequence for water suppression¹¹. Protein concentrations ranged from 50–150 µM in NMR buffer with 10% D₂O.

$R_2(^1\text{H})$ relaxation rates were assessed using the spin-echo sequence $90^\circ - \tau/2 - 180^\circ - \tau/2$ followed by the double spin echo sequence for water suppression¹¹. The peak intensities observed in two spectra recorded with $\tau = 1$ ms and 101 ms were converted into relaxation rates by assuming single exponential decays. The relaxation measurements were performed on a 600 MHz Bruker NMR spectrometer equipped with a cryoprobe.

Results

The unnatural amino acids of Fig. 1 are either commercially available (Tby and Tbf) or can be synthesized in a few steps (TMSmy, TMSf and dTbf, see the Supporting Information). Using cell-free synthesis reactions supplemented with suppressor-tRNA, *p*CNF-RS and unnatural amino acid, full-length protein samples were obtained in good yield for ubiquitin T66U, SrtA Y17U, Zipro *S81U and ERp29 S114U, when the unnatural amino acid was Tby, Tbf or TMSf. In contrast, we failed to incorporate TMSmy using the same system. This result may in part have arisen from the relatively low solubility of TMSmy in aqueous solution, but it is also possible that the larger size of the TMS-methylene group compared with the *tert*-butyl group renders TMSmy incompatible with the amino-acid binding pocket of the *p*CNF-RS enzyme.

The best expression yields were obtained with Tby and Tbf, where the final yields of purified protein were about 0.8 mg per 2 mL cell-free inner reaction mixture for ubiquitin T66U, SrtA Y17U and Zipro *S81U, and about 0.6 mg for ERp29 S114U. The yields were significantly lower with TMSf (between 0.3 and 0.5 mg per 2 mL inner reaction mixture). Expression of larger proteins with any of these unnatural amino acids proved difficult. No protein was obtained in attempts to produce T7 RNA polymerase (99 kDa) with amber stop codons in positions 312, 385, 623 or 846 by cell-free synthesis. Similarly, the Y115U and Y124U mutants of LeuRS (108 kDa) both failed to express under cell-free conditions with any of the unnatural amino acids and only LeuRS Y115Tby gave an acceptable yield *in vivo* (0.25 mg from 20 mL cell culture). *In vivo* expression of the amber stop mutants of ubiquitin and SrtA produced protein in good yield with Tby, but the yields were low with Tbf and no protein was obtained with TMSf.

The chemical shifts of the 9PS signal of the free amino acids in water at 25 °C are 1.34 ppm for Tby, 1.30 ppm for Tbf and 0.28 ppm for TMSf. Incorporation into a protein tends to change these chemical shifts depending on the specific environment. In general, the spectra of

ERp29 S114Tbf and SrtA Y17Tbf show that the 9PS signal of Tbf is about 0.1 ppm further upfield than the corresponding signal of Tby. The 9PS signal of TMSf was between 0.1 and 0.3 ppm (Fig. 2). In ubiquitin, the 9PS signals of all these amino acids are shifted upfield by about 0.4 ppm due to ring currents from Phe4, which is located near position 66 containing the unnatural amino acid residue.

The 9PS signals of TMSf tended to be broader than those of Tby and Tbf (Fig. 2), suggesting that the TMS group is less mobile than the *tert*-butyl group because of greater steric hindrance from the phenyl ring affecting the bond rotation rates. The methyl groups in Tbf and TMSf are in closer proximity to the aromatic ring protons than in Tby. To minimize dipole-dipole relaxation between the *tert*-butyl group in Tbf and the aromatic ring protons, we synthesized Tbf with deuterium substituting protons in the aromatic ring (dTbf). In all three proteins studied with Tbf and dTbf, the resulting 9PS signal was not much narrower than the signal from undeuterated Tbf (Fig. 2). The results showed that the relaxation rate of the 9PS signal of Tbf was generally greater than for Tby (Table 1), which may be attributed to the greater mobility of an O-*tert*-butyl group compared with an oxygen-free *tert*-butyl group. Use of dTbf versus Tbf hardly reduced the relaxation rates for the smaller proteins, but made a difference for ERp29. Despite the broad line width observed for the TMS resonance in the 1D ¹H-NMR spectrum of ERp29 (Fig. 2D), the apparent relaxation rate of this 9PS signal measured in a spin-echo experiment performed with and without 100 ms relaxation delay was comparable to that of dTbf (Table 1). This counterintuitive result may be explained by multi-exponential relaxation, which is a well-known feature of methyl groups and most pronounced under non-extreme narrowing conditions^{12–14}. Indeed, the resonance observed after a 301 ms delay of transverse relaxation was narrower than after a relaxation delay of 1 ms (11 Hz versus 6 Hz).

Quite generally, the relaxation rates increased with molecular mass, but the increase was not linear, as clearly demonstrated by the four proteins made with Tby. The width or relaxation rate of a 9PS signal thus is not a simple measure of the molecular weight. Unfortunately, LeuRS Y115U (like other proteins in the molecular weight range >100 kDa) could only be produced with Tby (Fig. S1) but not with Tbf or TMSf. Overall, our current set of data indicates that Tby and dTbf yield the narrowest 9PS signals, whereas the 9PS signal of TMSf, despite its broader width, comprises components that may relax equally or even more slowly.

As the 9PS signal of TMSf is in a spectral region with little signal overlap, it can more readily be tracked in ligand binding studies than the corresponding signal of Tby. This is illustrated by the Tby and TMSf samples of Zipro *S81U, where the 9PS signal of Tby overlaps with other, similarly narrow resonances of the protein, whereas the 9PS signal of TMSf is much better resolved at about -0.1 ppm (Fig. 3A). Titration of Zipro *S81TMSf with the generic

serine protease inhibitor 4-nitrophenyl-4-guanidinobenzoate hydrochloride (Fig. S2) greatly broadened the 9PS signal, indicating that the exchange rate of this inhibitor is in the slow to intermediate time regime (Fig. 3B). It is interesting that the TMSf residue in position *81 senses the binding of the inhibitor, which is known to bind to NS3 whereas residue *81 is in NS2B. Previous results obtained from ^{15}N -HSQC spectra of ^{15}N -labelled Zipro indicated that the C-terminal β -hairpin of NS2B, which comprises *Ser81, is dissociated from NS3 and mostly unaffected by the presence or absence of the inhibitor¹⁵.

Discussion

The present work demonstrates, for the first time, the incorporation of Tbf and TMSf into proteins as genetically encoded amino acids. We obtained these results by using the *p*CNF-RS enzyme, which has previously been shown to be poly-specific for a broad range of unnatural amino acids related to tyrosine⁵. Using cell-free synthesis, Tby, Tbf, dTbf and TMSf were successfully incorporated into human ubiquitin, *S. aureus* sortase A and rat ERp29. For proteins with molecular weights greater than 100 kDa, expression yields by cell-free synthesis proved to be too low to produce NMR samples, even for the 108 kDa protein LeuRS Y115U, which gave good expression yields with Tby *in vivo*. It is increasingly recognised that *E. coli* cell-free extracts work best for proteins smaller than 50 kDa¹⁶. Attempts to produce LeuRS with Tbf and TMSf *in vivo* failed. In general, *in vivo* protein expression requires significantly larger amounts of amino acids than cell-free synthesis¹⁷, prohibiting sample preparation with costly unnatural amino acids. The problem is amplified in the case of Tbf and TMSf, where *in vivo* expression of ubiquitin T66U and SrtA Y17U yielded little protein with Tbf and none with TMSf, suggesting that these amino acids are not easily imported across the cell membrane.

While *p*CNF-RS readily incorporates Tby, the cell-free incorporation yields of Tbf and, in particular, TMSf were lower, which would be expected for a lesser activity of the *p*CNF-RS with these amino acids. For difficult unnatural amino acids, it is well known that *E. coli* strains deficient in the release factor 1 (such as the B-95. Δ A strain) can, with unexpectedly high yields, read through an amber stop codon by misincorporation of a natural amino acid, if the suppressor tRNA is not efficiently loaded with the unnatural amino acid.^{10,18-23} The S30 extract used for our cell-free expressions was prepared from a cell-line, where the release factor 1 was present but had been tagged with chitin binding domains for selective removal prior to cell-free synthesis⁴. Nonetheless, we observed that also in this case full-length protein can be produced that does not contain the desired unnatural amino acid. Specifically, mass spectrometric analysis of tryptic digests of the ubiquitin T66TMSf sample revealed peptides containing either

glutamine or lysine in place of the unnatural amino acid. Therefore, the observation that the 9PS signal in the spectrum of ubiquitin T66TMSf (Fig. 2D) was about 10 times smaller than expected may be attributed to limited compatibility of the *p*CNF-RS enzyme with TMSf. The specific reasons for different incorporation yields in different proteins, however, are not understood.

In view of the greater rigidity of the *tert*-butyl group in Tbf than in Tby, the relaxation rate of the 9PS signal in Tbf was expected to be approximately proportional to the molecular mass of the protein. This was not observed. Potential explanations for this result include local mobility of the peptide segment harbouring the unnatural amino acid, different degrees of solvent exposure and multi-exponential relaxation effects.

It is interesting that the 9PS signal of TMSf can be broader than that of Tby, yet show slower $R_2(^1\text{H})$ relaxation rates. The effect was most clearly manifested for the 54 kDa dimer of ERp29, suggesting that it is governed by multi-exponential dipole-dipole relaxation, which is expected to be more pronounced at high molecular weight. Evidence for multi-exponential relaxation is also presented by the pronounced mismatch between the linewidths and $R_2(^1\text{H})$ relaxation rates measured. For example, none of the linewidths measured in the 1D ^1H -NMR spectra was below 5 Hz, which would correspond to a relaxation rate $>15\text{ s}^{-1}$, but the relaxation rates reported by the peak heights after transverse relaxation during 100 ms were significantly smaller (Table 1). The 9PS resonance of TMSf thus is advantageous not only for its chemical shift in a less crowded spectral region, but its slower transverse relaxation rates may also promote its detection in high-molecular weight systems using editing experiments, for example following ^{13}C labelling of the methyl groups.

Conclusion

In summary, we succeeded in genetic encoding of the amino acids Tbf and TMSf. The *tert*-butyl group of Tbf and the TMS group of TMSf produce ^1H -NMR resonances that can readily be identified without assigning any other NMR resonances. Their signals are sensitive reporters of the local environment, which can be used for ligand binding studies. Further optimization of the aminoacyl-tRNA synthetase may improve the incorporation yield of TMSf, which is attractive for its ^1H -NMR signal in a relatively well-resolved region of the protein NMR spectrum.

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Table 1. $R_2(^1\text{H})$ relaxation rates of the ^1H NMR signals of the *tert*-butyl and TMS groups in ubiquitin T66U, Sortase A Y17U, Erp29 S114U and LeuRS Y115U.^a

	MW	Tby	Tbf	dTbf	TMSf
ubiquitin T66U	8 kDa	4.2 s ⁻¹	5.3 s ⁻¹	4.2 s ⁻¹	2.8 s ⁻¹
sortase A Y17U	19 kDa	4.0 s ⁻¹	7.5 s ⁻¹	7.1 s ⁻¹	4.9 s ⁻¹
ERp29 S114U	54 kDa	8.4 s ⁻¹	11.4 s ⁻¹	7.3 s ⁻¹	7.0 s ⁻¹
LeuRS Y115U	108 kDa	23.3 s ⁻¹	n.d.	n.d.	n.d.

^a The transverse relaxation rates R_2 were determined at 600 MHz and 25 °C from two-time point measurements in a spin-echo experiment with relaxation delays differing by 100 ms. n.d. = not determined.

Figures

Fig. 1 Chemical structures of unnatural amino acids studied in the present work following incorporation into different proteins. (A) *tert*-butyltyrosine (Tby). (B) 4-(*tert*-butyl)phenylalanine (Tbf). (C) 4-(*tert*-butyl)phenylalanine-(phenyl-3,5-d₂) (dTbf). (D) 4-(trimethylsilyl)phenylalanine (TMSf). (E) 4-((trimethylsilyl)methoxy)phenylalanine (TMSmy).

Fig. 2 1D ^1H -NMR spectra of 0.1 mM solutions of ubiquitin T66U, sortase A Y17U and ERp29 S114U with the unnatural amino acids of Fig. 1. All spectra were measured at 25 °C on a 800 MHz NMR spectrometer. The panels (A) – (D) show spectra with Tby, Tbf, dTbf and TMSf, respectively. The chemical shifts of the *tert*-butyl and TMS groups in the ubiquitin T66U mutant are identified by a star. They are shifted due to ring currents from the side chain of Phe4 nearby. In the case of ubiquitin T66TMSf, this leads to overlap with the resonance of Leu50 $^{\delta}\text{CH}_3$, which is also at about -0.16 ppm.

Fig. 3 1D ^1H -NMR spectra of the Zika virus NS2B-NS3 protease with the mutation S81U in the NS2B co-factor. (A) Spectra of 0.1 mM solutions of the protease with either Tby (top panel) or TMSf (bottom panel) incorporated at position 81. Stars mark the 9PS signals of the *tert*-butyl and TMS groups, respectively. The spectra illustrate the improved resolution in the spectral region of the TMS group versus that of the *tert*-butyl group. (B) Spectra of a 0.05 mM solution of the Zika virus NS2B-NS3 protease mutant Ser81 TMSf. The arrow marks the resonance of the TMS group. Spectra are shown for different ratios of ligand (L) and protein (P) concentrations, where the ligand is the generic protease inhibitor 4-nitrophenyl-4-guanidinobenzoate hydrochloride.

Supporting Information

Genetically encoded amino acids with *tert*-butyl and trimethylsilyl groups for site-selective studies of proteins by NMR spectroscopy

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Fig. S1 1D ¹H-NMR spectrum of a 0.15 mM solution of archaeal *Mt*LeuRS Y115U with (top trace) and without (bottom trace) Tby at position 115. The signal of the *tert*-butyl group is identified by a star.

Fig. S2 Chemical structure of the protease inhibitor 4-nitrophenyl-4-guanidinobenzoate hydrochloride.

Chemical synthesis

The synthesis of 4-(trimethylsilyl)phenylalanine (TMSf), 4-(*tert*-butyl)phenylalanine-(phenyl-3,5-d2) (dTbf) and 4-((trimethylsilyl)methoxy)phenylalanine (TMSmy) were carried out as per Schemes S1, S2 and S3 respectively.

Scheme S1. Synthesis of TMSf.

Scheme S2. Synthesis of dTbf, major isomer.

Scheme S3. Synthesis of TMSmy.

General procedures and methods

Chemicals and solvents were purchased from standard suppliers and used without further purification. Analytical thin-layer chromatography analysis (TLC) was performed on pre-coated silica gel aluminium-backed plates (Merck Kieselgel 60 F₂₅₄). Visualisation was by examination under UV light at 254 nm, with subsequent staining by KMnO₄. Dry-flash column chromatography¹ was run using Davisil® P60 silica gel (40–63µm).

Optical rotation was measured in a JASCO P-2000 polarimeter at the sodium D line in a cell with 100 mm path length. Nuclear magnetic resonance (NMR) spectra were recorded on either a Bruker Ascend or a Bruker Avance Nanobay III 400 MHz Ultrashield Plus spectrometer at 400 MHz (¹H) and 101 MHz (¹³C). Chemical shifts (δ) are recorded in parts per million (ppm) downfield in relation to tetramethylsilane using the residual solvent as internal standard (CDCl₃, δ_H = 7.26 and δ_C = 77.16; D₂O, δ_H = 4.79).² Coupling constants (*J*) are reported in Hz with the following splitting abbreviations: singlet (s), doublet (d), triplet (t), quartet (q), broad (br), multiplet (m). High-resolution mass spectra (HRMS) were obtained from an Agilent 6224 TOF LC/MS Mass Spectrometer coupled to an Agilent 1290 Infinity. All data were acquired and reference mass corrected *via* a dual-spray electrospray ionisation (ESI) source.

Liquid chromatography mass spectrometry (LCMS) was performed with an Agilent 6120 Series Single Quad coupled to an Agilent 1260 Series HPLC. The following buffers were used: buffer A: 0.1% formic acid in H₂O; buffer B: 0.1% formic acid in MeCN. The following gradient was used with a Poroshell 120 EC-C18 50 x 3.0 mm 2.7 micron column, and a flow rate of 0.5 mL/min and total run time of 5 min: 0–1 min 95% buffer A and 5% buffer B, from 1–2.5 min up to 0% buffer A and 100% buffer B, held at this composition until 3.8 min, 3.8–4 min 95% buffer A and 5% buffer B, held until 5 min at this composition. Mass spectra were acquired in positive and negative ion mode with a scan range of 100-1000 *m/z*. UV detection was carried out at 214 and 254 nm. All retention times (*R*_t) are quoted in min.

4-Nitrobenzyl 2-((*tert*-butoxycarbonyl)amino)acrylate.³ DIPEA (17.8 mL, 102 mmol) and 4-nitrobenzyl bromide (22.1 g, 102 mmol) were added sequentially to a suspension of Boc-Ser-OH (20.0 g, 97.5 mmol) in EtOAc (500 mL), under N₂. The reaction mixture was heated at 75 °C for 3 days and was then allowed to cool RT. The organic suspension was washed with H₂O (4 × 200 mL), brine and dried (Na₂SO₄). Solvent was removed under reduced pressure to give a pale yellow solid (*N*-(*tert*-butoxycarbonyl)-L-serine *p*-nitrobenzyl ester), which was used without purification. The solid was dissolved in CHCl₃ (780 mL) and copper(I) chloride (977 mg, 9.87 mmol) followed by EDC hydrochloride (37.9g, 197 mmol) were added. The mixture was stirred, under N₂, for 19 h at RT. The reaction mixture was quenched with brine (800 mL) and then the biphasic mixture was filtered through Celite. The layers were separated and the aqueous was extracted with CHCl₃ (2 × 400 mL). The combined organics were dried (Na₂SO₄) and solvent was removed under reduced pressure. The crude material was purified by silica dry-flash chromatography, eluting with a gradient of 10–50% EtOAc in petroleum benzine to give the title compound as a white solid (29.3 g, 93% yield over 2 steps). ¹H NMR (400 MHz, CDCl₃) δ 8.28 – 8.21 (m, 2H), 7.58 – 7.50 (m, 2H), 6.98 (br s, 1H), 6.24 (s, 1H), 5.82 (d, *J* = 1.4 Hz, 1H), 5.35 (s, 2H), 1.48 (s, 9H). MS (ES) *m/z*: 345 [M+Na]⁺. LCMS: *R*_t = 3.60 min; area 97%.

(4-Iodophenyl)trimethylsilane.⁴ $^n\text{BuLi}$ (31 mL, 61.2 mmol, 2 M solution in cyclohexane) was added dropwise to a cooled ($-65\text{ }^\circ\text{C}$) solution of 1,4-diiodobenzene (20.0 g, 60.6 mmol) in Et_2O (60 mL), under N_2 . Reaction temperature was kept below ($-55\text{ }^\circ\text{C}$) during the addition. Once the addition was complete (15 min), the dry ice/acetone bath was removed and the mixture was allowed to warm to $12\text{ }^\circ\text{C}$ over 1.5 h. The reaction mixture was cooled to $-65\text{ }^\circ\text{C}$ and TMSCl (8.46 mL, 66.7 mmol) was added dropwise over 5 min, while maintaining reaction temperature below $-55\text{ }^\circ\text{C}$. The dry ice/acetone bath was removed and the reaction was allowed to warm to RT over 4 h. The reaction mixture was cooled to $5\text{ }^\circ\text{C}$, quenched with a 10% aqueous solution of Na_2CO_3 (100 mL) and then the biphasic mixture was filtered under suction. The filter was washed with Et_2O (60 mL) and the filtrate layers were separated. The organic layer was washed with 10% aqueous Na_2CO_3 (100 mL), brine, dried (Na_2SO_4) and solvent was carefully removed under reduced pressure at $40\text{ }^\circ\text{C}$. The crude material was purified by silica dry-flash chromatography, eluting with petroleum benzine to give the title compound as an orange oil (15.5 g, 93%). ^1H NMR (400 MHz, CDCl_3) δ 7.72 – 7.67 (m, 2H), 7.27 – 7.22 (m, 2H), 0.26 (s, 9H). ^{13}C NMR (CDCl_3) δ 140.0 (C), 136.9 (CH), 135.2 (CH), 95.8 (C), -1.14 (CH_3).

4-Nitrobenzyl-2-((tert-butoxycarbonyl)amino)-3-(4-(trimethylsilyl)phenyl)acrylate.

Degassing was achieved by passing a stream of nitrogen through the solvent for 15 min prior to use. 4-Nitrobenzyl 2-[(tert-butoxycarbonyl)amino]acrylate (6.73 g, 20.9 mmol) followed by NaHCO_3 (3.83 g, 45.5 mmol) was added to a mixture of tetrabutylammonium chloride monohydrate (6.18 g, 20.9 mmol) and (4-iodophenyl)trimethylsilane (5.24 g, 19.0 mmol) in degassed DMF (75 mL). $\text{Pd}(\text{OAc})_2$ (298 mg, 1.33 mmol) was added to the reaction mixture, which was degassed for 1 min and then heated at $80\text{ }^\circ\text{C}$, under N_2 , for 26 h. The mixture was allowed to cool to RT, quenched with H_2O (400 mL) and the aqueous was extracted with

EtOAc (3 × 200 mL). The combined organics were washed with H₂O (2 × 200 mL), brine, dried (Na₂SO₄) and solvent was removed under reduced pressure. The crude material was purified by silica dry-flash chromatography, eluting with a gradient of 5–15% EtOAc in petroleum benzine to give the title compound as a pale yellow solid (5.73 g, 64%). ¹H NMR (400 MHz, CDCl₃) δ 8.27 – 8.21 (m, 2H), 7.62 – 7.56 (m, 2H), 7.56 – 7.52 (m, 2H), 7.52 – 7.48 (m, 2H), 7.28 (s, 1H), 6.12 (br s, 1H), 5.37 (s, 2H), 1.41 (s, 9H), 0.27 (s, 9H). ¹³C NMR (CDCl₃) δ 165.3 (C), 153.1 (C), 148.0 (C), 143.1 (C), 143.1 (C), 134.0 (C), 133.8 (CH), 131.4 (CH), 129.0 (CH), 128.7 (CH), 124.9 (C), 124.0 (CH), 81.3 (C), 65.9 (CH₂), 28.3 (CH₃), -1.14 (CH₃). MS (ES) *m/z*: 469 [M-H]. LCMS: *R*_t = 3.86 min; area 95%. HRMS: calcd for C₂₄H₃₀N₂O₆Si + Na⁺, 493.1765; found (ES, [M+Na]⁺ obsd), 493.1776.

2-((tert-Butoxycarbonyl)amino)-3-(4-(trimethylsilyl)phenyl)propanoic acid. 10% Pd/C (1.44 g) was added to a solution of 4-nitrobenzyl-2-((tert-butoxycarbonyl)amino)-3-(4-(trimethylsilyl)phenyl)acrylate (14.7 g, 31.2 mmol) in MeOH (375 mL) and CH₂Cl₂ (75 mL), under N₂. The reaction flask was briefly evacuated, then backfilled with hydrogen *via* balloon, and this process was repeated a further two times. The mixture was then stirred at RT for 22 h. The reaction mixture was filtered through Celite and the pad was washed with MeOH (2 × 250 mL). The filtrate was evaporated under reduced pressure. The resultant residue was dissolved in Et₂O (500 mL) and then washed with H₂O acidified to pH 1 with conc. HCl (4 × 200 mL), brine, dried (Na₂SO₄) and solvent was removed under reduced pressure to give the title compound as a pale brown foam (9.34 g, 89%). ¹H NMR (400 MHz, CDCl₃) δ 7.46 (d, *J* = 7.9 Hz, 2H), 7.18 (d, *J* = 7.2 Hz, 2H), 4.93 (br d, *J* = 7.7 Hz, 1H), 4.69 – 4.56 (m, 1H), 3.20 (dd, *J* = 13.8, 5.2 Hz, 1H), 3.07 (dd, *J* = 13.8, 6.4 Hz, 1H), 1.42 (s, 9H), 0.25 (s, 9H). ¹³C NMR (CDCl₃) δ 176.1 (C), 155.6 (C), 139.2 (C), 136.4 (C), 133.8 (CH), 128.9 (CH), 80.5 (C), 54.4 (CH), 37.9 (CH₂), 28.4 (CH₃), -0.98 (CH₃). MS (ES) *m/z*: 336 [M-H]. HRMS: calcd for C₁₇H₂₇NO₄Si + Na⁺, 360.1602; found (ES, [M+Na]⁺ obsd), 360.1602.

2-Amino-3-(4-(trimethylsilyl)phenyl)propanoic acid hydrochloride (TMSf hydrochloride). A solution of 4 M HCl in dioxane (100 mL) was added to a solution of 2-[(tert-butoxycarbonyl)amino]-3-[4-(trimethylsilyl)phenyl]propanoic acid (9.34 g, 27.7 mmol) in dioxane (100 mL). The mixture was stirred at RT for 16 h and then filtered. The solid was washed with Et₂O (2 × 40 mL) and dried under suction. Residual solvent was then removed under reduced pressure to give the title compound as a white solid (5.72 g, 75%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.43 (br s, 3H), 7.48 (d, *J* = 8.0 Hz, 2H), 7.27 (d, *J* = 8.0 Hz, 2H), 4.15 (t, *J* = 6.3 Hz, 1H), 3.12 (d, *J* = 6.3 Hz, 2H), 0.23 (s, 9H). ¹³C NMR (CDCl₃) δ 170.3 (C), 138.4 (C), 135.6 (C), 133.4 (CH), 128.9 (CH), 53.0 (CH), 35.7 (CH₂), -1.13 (CH₃). MS (ES) *m/z*: 238 [M+H]⁺. HRMS: calcd for C₁₂H₁₉NO₂Si + H⁺, 238.1258; found (ES, [M+H]⁺ obsd), 238.1260.

(S)-2-Amino-3-(4-(tert-butyl)phenyl-3,5-d₂)propanoic acid (dTbf), major isomer. 4-tert-Butyl-L-phenylalanine (500 mg, 2.26 mmol) was dissolved in D₂O (12 mL) with the addition of 40% w/w NaOD in D₂O (12 drops). The amino acid was precipitated with the addition of 96-98% w/w D₂SO₄ in D₂O and collected by filtration. The collected solid was dissolved in a mixture of D₂SO₄ (3.5 g) and D₂O (0.5 g) and heated at 90 °C, under N₂, for 4 h. Ice (2.5 g) followed by ammonia (~ 7.5 mL) was added until the amino acid precipitated. The solid was collected by filtration and precipitated from D₂O by adding, sequentially, NaOD followed by D₂SO₄ as described above. The collected solid was washed with D₂O (2 × 1 mL) and then dried under reduced pressure over KOH to give the title compound as a white solid (340 mg, 67%). The ¹H NMR spectrum described below showed 92% deuteration of the 3,5 phenyl protons and 45% deuteration of the 2,6 phenyl protons. This assignment was unambiguously confirmed by HSQC and HMBC NMR experiments. ¹H NMR (400 MHz, D₂O + 1 drop NaOD) δ 7.45 – 7.42 (m, 0.15H), 7.22 (s, 1.11H), 3.46 (dd, *J* = 7.5, 5.4 Hz, 1H), 2.96 (dd, *J* = 13.6, 5.4 Hz, 1H), 2.77 (dd, *J* = 13.6, 7.5 Hz, 1H), 1.27 (s, 9H). ¹³C NMR (D₂O + 1 drop NaOD) δ 182.5 (C),

149.9 (C), 135.4 (t, J_{C-D} = 8.6 Hz, C), 129.3 (CH), 57.4 (CH), 40.2 (t, J_{C-D} = 5.3 Hz, CH₂), 33.7 (C), 30.5 (CH₃). MS (ES) m/z : 224 [M+H]⁺. LCMS: R_t = 2.88 min; area 100%. HRMS: calcd for C₁₃H₁₇D₂NO₂ + H⁺, 224.1614; found (ES, [M+H]⁺ obsd), 224.1614.

Methyl

(S)-2-((tert-butoxycarbonyl)amino)-3-(4-

((trimethylsilyl)methoxy)phenyl)propanoate. (Chloromethyl)trimethylsilane (520 μ L, 3.72 mmol) was added dropwise (over 5 min) to a mixture of Boc-Tyr-OMe (1.00 g, 3.39 mmol) and K₂CO₃ (515 mg, 3.72 mmol) in anhydrous DMF (4 mL), at 80 °C under N₂. The reaction mixture was then heated at 90 °C for 5 h. The mixture was then allowed to cool to 80 °C, further K₂CO₃ (558 mg, 4.04 mmol) and (chloromethyl)trimethylsilane (520 μ L, 3.72 mmol) was added and the reaction was heated at 90 °C for 4 h. At this point the mixture was allowed to cool to 80 °C, additional K₂CO₃ (538 mg, 3.89 mmol) and (chloromethyl)trimethylsilane (520 μ L, 3.72 mmol) was added and the reaction was heated at 90 °C for 15 h. Once more the reaction was allowed to cool to 80 °C, additional K₂CO₃ (540 mg, 3.91 mmol) and (chloromethyl)trimethylsilane (520 μ L, 3.72 mmol) was added and the mixture was heated at 90 °C for 2 h. The reaction was then allowed to cool to RT, quenched with H₂O (25 mL) and the aqueous was extracted with Et₂O (4 $\times$ 25 mL). The combined organics were washed with H₂O (2 $\times$ 25 mL), brine, dried (Na₂SO₄) and solvent was removed under reduced pressure. The crude material was purified by silica dry-flash chromatography, eluting with a gradient of 5–15% EtOAc in petroleum benzine to give the title compound as a colourless oil (983 mg, 76%). $[\alpha]_D^{27}$ = +14.5 (c = 1.00, CHCl₃). ¹H NMR (400 MHz, CDCl₃) δ 7.01 (d, J = 8.6 Hz, 2H), 6.91 – 6.84 (m, 2H), 4.94 (br d, J = 7.9 Hz, 1H), 4.53 (dt, J = 7.9, 5.8 Hz, 1H), 3.71 (s, 3H), 3.53 (s, 2H), 3.04 (dd, J = 14.0, 5.8 Hz, 1H), 2.99 (dd, J = 14.0, 5.8 Hz, 1H), 1.42 (s, 9H), 0.13 (s, 9H). ¹³C NMR (CDCl₃) δ 172.6 (C), 160.8 (C), 155.3 (C), 130.2 (CH), 127.5 (C), 114.3 (CH), 80.0 (C), 61.2 (CH₂), 54.7 (CH), 52.3 (CH₃), 37.5 (CH₂), 28.4 (CH₃), -3.02 (CH₃). MS (ES) m/z : 404 [M+Na]. LCMS: R_t = 3.72 min; area 100%. HRMS: calcd for C₁₉H₃₁NO₅Si + Na⁺, 404.1864; found (ES, [M+Na]⁺ obsd), 404.1866.

(S)-2-amino-3-(4-((trimethylsilyl)methoxy)phenyl)propanoic acid hydrochloride (TMSmy hydrochloride). A solution of 2 M NaOH (4 mL) was added to a solution of methyl (S)-2-((tert-butoxycarbonyl)amino)-3-(4-((trimethylsilyl)methoxy)phenyl)propanoate (938 mg, 2.46 mmol) in THF (4 mL) and the mixture was stirred at 25 °C for 19 h. Additional THF (8 mL) and 2 M NaOH (8 mL) was added and the mixture was heated at 65 °C for 3 h. The reaction was diluted with H₂O (25 mL) and then washed with Et₂O (2 × 25 mL). The aqueous was acidified to pH 4 with 6 M HCl and then extracted with EtOAc (4 × 25 mL). The combined organics were washed with brine, dried (Na₂SO₄) and solvent was removed under reduced pressure. The crude material was partially purified by silica dry-flash chromatography, eluting with a gradient of 10–80% EtOAc in petroleum benzene. This material was dissolved in EtOAc (25 mL) and 1 M NaOH (25 mL) was added. Layers were separated and the aqueous was extracted with EtOAc (25 mL). The combined organics were washed with brine, dried (Na₂SO₄) and solvent was removed under reduced pressure. The resultant foam was dissolved in EtOAc (25 mL), H₂O (25 mL) was added and the aqueous was acidified to pH 1 with 1 M HCl. Layers were separated and the aqueous was extracted with EtOAc (2 × 25 mL). The combined organics were washed with brine, dried (Na₂SO₄) and solvent was removed under reduced pressure to give the acid as a colourless oil (703 mg). The acid (670 mg, 1.82 mmol) was dissolved in dioxane (8 mL) and a solution of 4 M HCl in dioxane (8 mL) was added. The mixture was stirred at RT for 14 h and then filtered. The solid was washed with Et₂O (2 × 4 mL) and then dried under suction give the title compound as a white solid (305 mg, 55% yield over 2 steps). $[\alpha]^{27}_D = -0.3$ ($c = 1.00$, MeOH). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.41 (br s, 2H), 7.16 (d, $J = 8.7$ Hz, 2H), 6.91 (d, $J = 8.7$ Hz, 2H), 4.06 (t, $J = 6.2$ Hz, 1H), 3.58 (s, 2H), 3.07 (d, $J = 6.2$ Hz, 2H), 0.11 (s, 9H). ¹³C NMR (DMSO-*d*₆) δ 170.4 (C), 160.3 (C), 130.5 (CH), 126.3 (C), 114.1 (CH), 60.7 (CH₂), 53.4 (CH), 34.8 (CH₂), -3.07 (CH₃). MS (ES) m/z : 268 [M+H]⁺. LCMS: $R_t = 3.02$ min; area 100%. HRMS: calcd for C₁₃H₂₁NO₃Si + H⁺, 268.1363; found (ES, [M+H]⁺ obsd), 268.1367.

NMR Spectra

References

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