Article

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A Peptidyl-Prolyl Model Study: How Does the *Electronic Effect* Influence the Amide Bond Conformation?

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The authors declare no competing financial interest.

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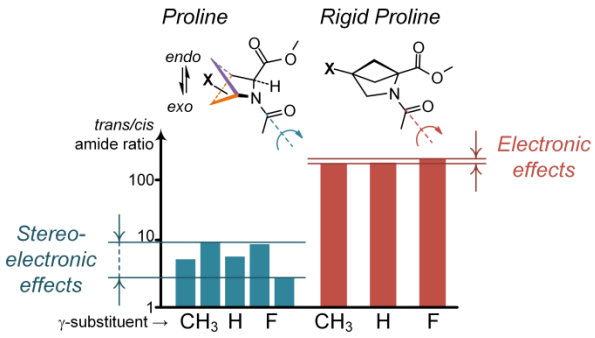
Proline
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γ-substituent →
CH3 H F
CH3 H F

Abstract

The triple-helical structure of collagen, the most abundant protein in animal body, owes its stability to post-translationally installed hydroxyl groups at position 4 of prolyl residues. To shed light on the nature of this phenomenon we have examined the influence of the 4-substituent on the amide isomerism in peptidyl-prolyl analogues. The rigid bicyclic skeleton of 2,4-methanoprolines allowed us to follow the through-bond impact of the substituent group (*electronic effect*), without the side-chain conformation being affected by a *stereoelectronic effect*. These proline analogues were prepared by [2+2] photocycloaddition of (2-allylamino)acrylic acid derivatives. Subsequent pK_a studies demonstrated a remarkable *electronic effect* of the 4-fluorine substitution, while the effect of the 4-methyl group was negligible. The *trans/cis* amide ratio was measured in model compounds under low temperature conditions. The observed prevalence for a *trans*-amide is extraordinary, and in this regard 2,4-methanoproline is closer to primary α-amino acids than to proline. The amide rotation barrier was also found to be significantly reduced: as equivalent to 3-4 orders of magnitude higher isomerization velocities when compared to *N*-acetyl-prolyl. Finally, our results indicate that the *electronic effect* only affects the kinetics of the amide isomerization, but not the thermodynamic prevalence for the *trans*-rotamer.

Introduction

Collagen is the most abundant protein in mammals, and its malfunctions are related to a variety of diseases.[1] Perhaps, the historically best known among these diseases is scurvy, which is caused by a lack of vitamin C, essential for maintaining a correct hydroxylation rate of repetitive prolyl-prolyl-glycyl motifs in procollagen.[2,3] This post-translational modification yields (*4R*)-hydroxyproline (**1**, Chart 1) residues in positions preceding glycine. As a result, the stability of triple helical arrangements increases and the melting point of collagen is adjusted above body temperature, ensuring the correct integrity of connective tissues.[4]

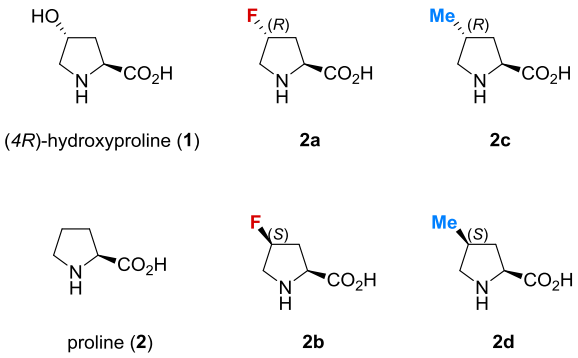
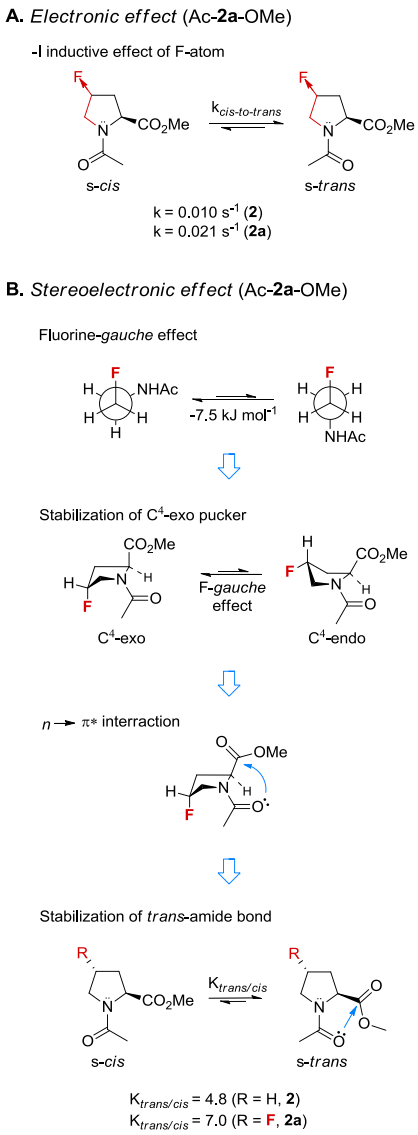


Chart 1 Structures of (*4R*)-hydroxyproline (**1**), proline (**2**) and diastereomeric 4-fluoro- (**2a**, **2b**) and 4-methylprolines (**2c**, **2d**).

The essential role of prolyl hydroxylation had not been well understood until the late 1990s, when Raines and colleagues discovered that incorporation of (*4R*)-fluoroproline (**2a**) into repetitive collagen mimicking sequences results in even higher thermal stability as compared to analogous sequences containing hydroxyproline (**1**).[5] It therefore became apparent that neither hydrogen bond nor water network stabilization is promoted by prolyl hydroxylation in collagen, but that rather an electron withdrawing substituent enhances the stability of the structure due to

its *electronic effect*. The latter can be conceived as a through-bond interaction of a side chain substituent to the backbone groups, and it is manifested by a significant reduction of the amide bond isomerization barriers (Scheme 1A).[6]

This simple hypothesis was later confronted with the fact that incorporation of (*4S*)-fluoroproline (**2b**) in the glycine-preceding positions decreases the stability of the triple helical motif dramatically.[7] Another remarkable finding was that the incorporation of 4-methylprolines (**2c**, **2d**) into collagen-mimicking sequences impacts the triple helix stability to a similar extent as do 4-hydroxy- or 4-fluoroprolines.[8] Yet, the effect of methylprolines exhibits a reverse chiral bias as compared to fluoroprolines: it is the (*4S*)-isomer (**2d**) which stabilizes the triple helix when incorporated in front of glycine residues in the sequence, whereas (*4R*)-isomer (**2c**) exhibits a destabilizing effect. Based on these observations, the stabilizing effect of the (*4R*)-fluoroproline was assigned to a *stereoelectronic effect*,[9] which is based on the fluorine *gauche*-effect.[10] A fluorine *gauche* conformation stabilizes the C^4-*exo* pucker in **2a** (Scheme 1B), which promotes an $n \rightarrow \pi^*$ interaction between an amide oxygen lone pair and the upstream carbonyl group. The latter interaction is eventually responsible for a thermodynamic stabilization of the *trans* amide bond. Stabilization of both the prolyl *exo*-pucker and the *trans*-amide bond are critical for the folding stability and packing of collagen triple helical chains.[11]



Scheme 1. Schematic illustration of the *electronic* (**A**) and *stereoelectronic effect* (**B**) on the (*4R*)-fluoroproline structure.[a]

[a] The numeric data are taken from ref.[12] (proline and analogues) and ref.[10] (*gauche*-effect).

The discovery of the *stereoelectronic effect* provided an essential understanding of the polyproline fold, and this concept was later employed for the construction of clickable,[13] pH-switchable,[14] and hydrophobic[15] scaffolds based on stable all-*trans* amide polyproline II type

helical structures.[16,17] The ubiquity of the n→π* interaction in protein structures has been demonstrated,[18] and it was shown that the n→π* interaction is competitive with hydrogen bonding, since both require an electron density donation from the carbonyl oxygen atom.[19] Numerous proline-to-fluoroproline substitutions have been reported, and these showed adverse effects on the folding and stability of proteins and protein domains due to their impact on the *trans/cis* amide isomerism.[20,21] Furthermore, effects onto the thermodynamic stability of amide bond conformers have been identified for a variety of 4-substituted prolines,[22] providing an important toolbox for the functionalization of proteins and peptide motifs.

Nevertheless, it is still not possible to completely discriminate between *electronic* and *stereoelectronic effects* based on the existing data on proline analogues. For instance, we recently demonstrated that the carboxylic group in *N*-acetylated (*4R*)-fluoroproline (Ac-**2a**) is considerably more acidic than in the respective (*4S*)-isomer (Ac-**2b**).[23] The higher electrophilicity of the carboxylic group in **2a** can also be inferred from the remarkably high velocity of the peptidyl transfer reaction with this amino acid measured on the ribosome (about 150-fold increase compared to proline).[24] Since n→π* interaction is a donation, one could expect that a more electrophilic C-terminal carboxyl group in **2a** may enhance the strength of this interaction, and this can lead to some *trans*-amide stabilization following the *electronic effect* in addition to the existent *stereoelectronic effect*.

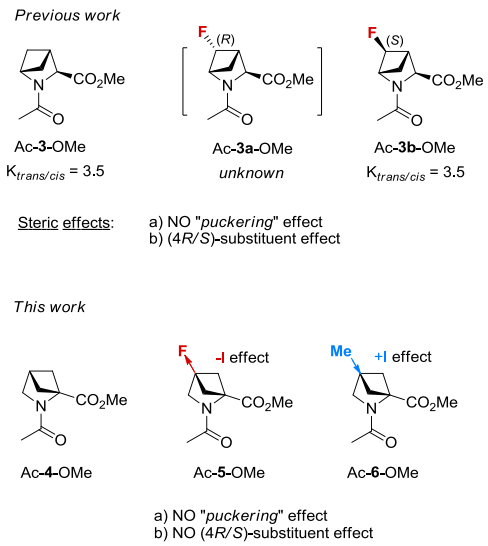


Chart 2 Model compounds that enable for elucidation of the *electronic effect* on prolyl amide isomerisation.

It is therefore desirable to discriminate between the two possible stabilization effects. An interesting approach, which addressed this issue was previously presented by Krow, Raines, and co-workers.[25] In particular, they prepared compound Ac-**3b**-OMe and demonstrated that its amide equilibrium constant was nearly identical to that of parent non-fluorinated compound Ac-**3**-OMe (Chart 2). Although, this finding provided an important indication for the insignificance of an *electronic effect* on the amide rotameric ratio, it should be kept in mind that conformationally restricted compound **3b** has a rigidly fixed antiperiplanar orientation of the fluorine substituent with respect to the nitrogen atom. Compound Ac-**3a**-OMe which would mimic the *gauche*-conformation as seen in 4-fluoroprolines was not studied. In addition, no numerical values (such as pK_a) were obtained and it remains therefore unclear, to which extent the 4-substituent influences electronic properties of the amino acid functional groups in this model system.

This motivated us to design a model study based on the 2,4-methanoproline structure (Chart 2). We assumed that the rigid 2-azabicyclo[2.1.1]hexane skeleton would allow for observation of

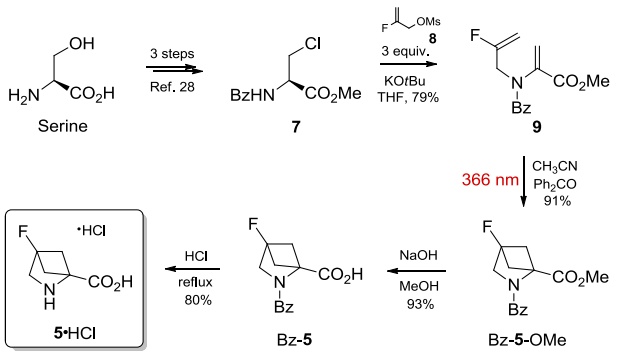
the pure *electronic effect* of a 4-substituent, since the amino acids are devoid of any puckering transition and any chiral bias. We therefore performed the synthesis of the model compounds Ac-**4**/**5**/**6**-OMe and report herein on the properties of the amide bond formed in these structures.

Results and Discussion

Synthesis

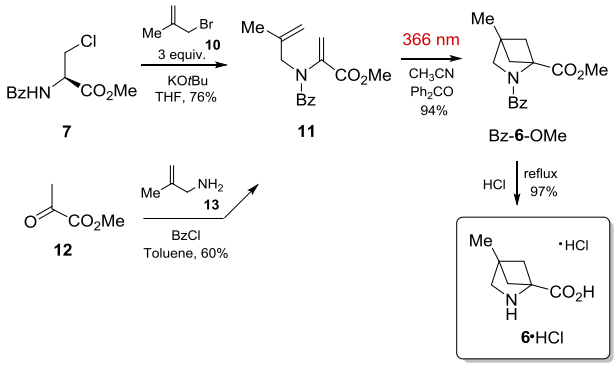
While parent 2,4-methanoproline (**4**) is well known, and reported to be an anti-insect metabolite produced in some plants,[26,27] methanoproline analogues **5** and **6** had to be synthesized. Recent advances in photochemical transformations allowed for the preparation of these compounds,[28,29] and we have optimized a gram-scale synthesis of derivatives bearing electron withdrawing (fluorine atom) and donating (methyl group) substituents in position 4 of the bicyclic skeleton.

In 2009, we already reported on the synthesis of amino acid **5** on a small scale (20 mg).[30] The most problematic step of the synthesis was the allylation of **7** to **9**, and only a 25% yield was achieved. We therefore performed an optimization of this step, and found that the use of purified reagent **8**[31] and an increase of its amount to 3.0 equiv. led to a much better yield of the target amide **9** (79%, Scheme 2). Lower amounts of alkylating agent gave significantly lower yields, whereas a larger excess of **8** did not result in any further improvement. Subsequently, the intramolecular crossed [2+2] photocycloaddition of alkene **9** to proline derivative Bz-**5**-OMe was smoothly performed under 366 nm irradiation using benzophenone as the sensitizer. Saponification of the ester group in Bz-**5**-OMe produced free acid Bz-**5**, which was transformed into the target amino acid **5**·HCl using acidic hydrolysis on gram scale.



Scheme 2. Optimized synthesis of the amino acid **5**·HCl.

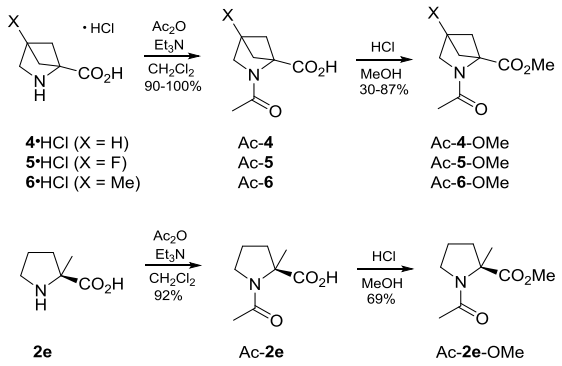
Synthesis of amino acid **6** was performed analogously (Scheme 3). Alkylation of compound **7** with 3.0 equiv. of methallyl bromide (**10**) afforded the product **11** in 76% yield. Alternatively, diene **11** was synthesized from methyl pyruvate (**12**) and amine **13** in 60% yield. The key transformation – intramolecular [2+2] photocycloaddition of **11** – was easily performed at 366 nm giving the bicyclic compound Bz-**6**-OMe in 94% yield. Simultaneous hydrolysis of both amide and ester groups in Bz-**6**-OMe gave the target amino acid **6**·HCl also on gram scale.



Scheme 3. Synthesis of the amino acid **6**·HCl.

With the amino acids in hand, the model compounds for further physicochemical characterizations were prepared using standard methods (Scheme 4). In addition, analogous

derivatives of 2-methylproline (**2e**) were prepared for comparison. 2-methylproline is a well studied α-substituted proline analogue,[32,33,34] and it should represent a case of a quaternary proline for comparison with 2,4-methanoprolines which are also quaternary amino acids.



Scheme 4. Synthesis of the model compounds for physicochemical characterizations.

The electronic effect

The characterization of the electronic effects in 2,4-methanoprolines was then attempted using numerical parameters. Perhaps the most straightforward measurable value that accesses the electronic influence of amino acid substituents on the functional groups is the acidity. We therefore measured acidity constants for the ammonium and for the carboxylic group of 4-substituted 2,4-methanoprolines. The values we reported previously for 4-substituted prolines are included for comparison.[23,24]

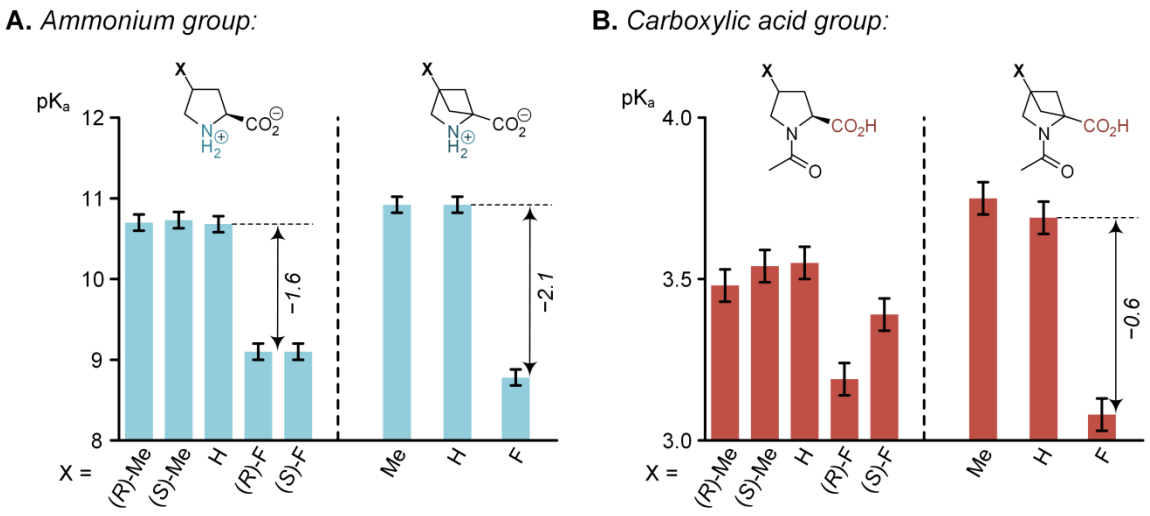


Figure 1 Acidity of the functional groups as measured by NMR in aqueous medium at 298 K.

Analysis of the obtained values (Figure 1) clearly indicates the absence of any detectable electronic influence of the 4-methyl group on both functional groups of the examined amino acid. In contrast to that, a 4-fluorine substituent exhibits a significant electron-withdrawing effect. In the case of 2,4-methanoprolines, the reduction of the basicity (N-terminal) was by 2.1 pK_a units (Figure 1A), and the increase in the carboxylic acid acidity (C-terminal) was by about 0.6 pK_a units (Figure 1B). Thus, a significant increase of the C-terminal carboxyl electrophilicity can be expected, and this increase should correlate with an *electronic effect* on the *trans/cis* equilibrium.

The amide isomerism

It is commonly accepted that the *trans/cis* amide propensities for peptidyl-prolyl modifications can be characterized using simple model compounds. The most extensively exploited models are methyl esters of *N*-acetyl amino acids (Ac-AA-OMe, where AA is the amino acid).[11,12] We thus set up to measure *trans/cis* amide equilibrium values for Ac-**4**/**5**/**6**-OMe

in deuterium oxide solution by NMR. In particular, our aim was to examine whether or not the *trans*-amide stabilizing n→π* interaction was substantially affected by an *electronic effect*.

Curiously, the equilibrium population measurements at the standard temperature (298 K) were hampered by the observation that the barriers of the amide rotation seemed to be extremely low for 2,4-methanoprolines. Previously, a model compound analysis was performed in the 1980s, where it was reported that 2,4-methanoproline derivatives exist as *trans*-amides.[35] However, no numerical characterization was provided, and it was therefore not clear whether this observation was a result of a large *trans*-amide dominance or whether the minor *cis*-amide could not be detected due to a fast amide equilibration on the NMR time scale. Our study revealed that both scenarios appeared to be valid. We found that lowering the temperature of the examined model substance solutions sufficiently separated the *cis*-amide resonances from the *trans* signals enabling the direct observation of both rotameric species. Subsequently, we determined the rotameric ratios and the interconversion barriers for the 2,4-methanoproline derivatives (Figure 2).

Analysis of the obtained values indicates that for all three examined 2,4-methanoprolines the prevalence of the *trans*-amide rotameric form is massive, giving rise to *trans/cis* ratios in the range of 140-180 (in aqueous medium at 276 K, Figure 2A). A high *trans/cis* ratio for 2,4-methanoproline derivatives was also observed in methanol-d_4 solutions (See Table S3 in SI). To the best of our knowledge, such an extraordinary dominance of one rotameric form has never been reported for a proline analogue. When converted to free energy, the values found in Ac-**4**/**5**/**6**-OMe (−11.2 to −11.9 kJ mol^{-1}) are far greater than that for proline (−4.0 ± 0.1 kJ mol^{-1}), and they already approach the *trans*-amide preferences of standard α-amino acid amides (e.g. −12.6 ± 0.3 kJ mol^{-1} for Ac-Gly-OMe at 298 K).[23]

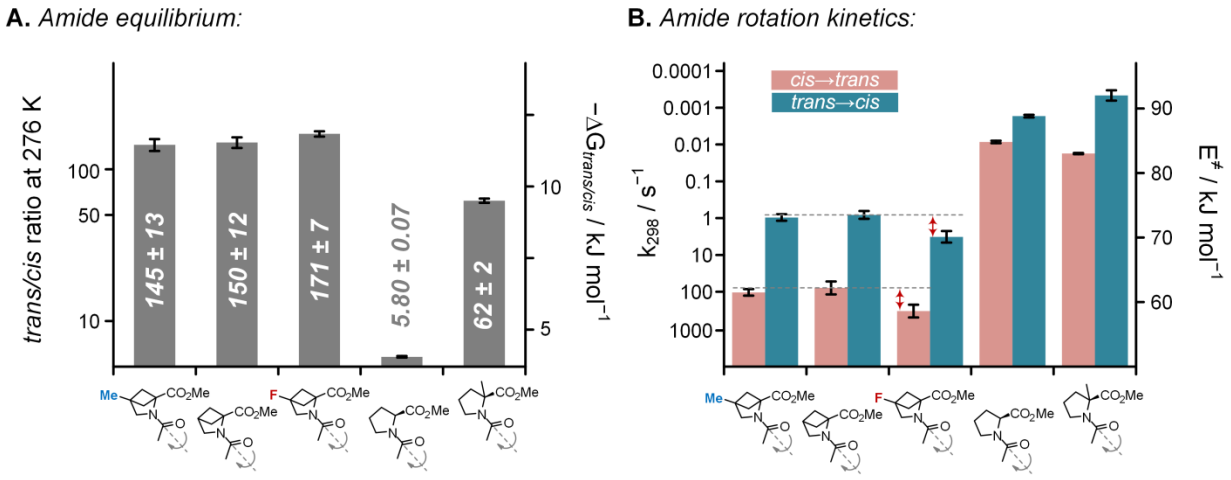


Figure 2 Equilibrium and kinetic parameters of the *trans*/*cis* amide isomerism in Ac-AA-OMe solution in deuterium oxide.[a]

[a] $K_{trans/cis}$ values were determined at 276 K and converted to the free energy values. The rotational barriers were determined at 276 (for Ac-**4**/**5**/**6**-OMe) and 310 K (for Ac-**2**/**2e**-OMe) and were converted to rate constants using Eyring equation assuming purely enthalpic energy of isomerization. For details see Tables S3 and S4.

Further analysis of the obtained values indicated almost no influence of the 4-substituent on the *trans/cis* ratio. For instance, we expected a significant *electronic effect* from 4-fluorine substitution based on the analysis of the pK_a values earlier. Indeed, for the Ac-**5**-OMe we found a higher prevalence of the *trans*-rotamer which was however marginal and close to the measurement error: −0.3 ± 0.3 kJ mol^{-1} and −0.4 ± 0.3 kJ mol^{-1} in deuterium oxide and methanol-d_4 solutions, respectively. In contrast to this observation, the *stereoelectronic effect* in analogous 4-substituted prolines promotes more significant changes, which extend from −1.2 to +1.7 kJ mol^{-1} in relative stabilization of the *trans*-rotamer (Figure 3). Therefore, the thermodynamic influence of the pure *electronic effect* in our model system is minimal at best.

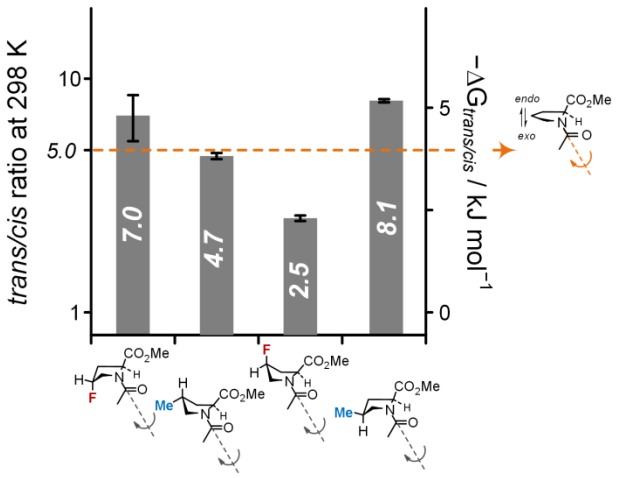


Figure 3 Manifestation of the *stereoelectronic effect* in 4-substituted proline analogues (deuterium oxide solution, 298 K). The data taken from ref.[12,15] (see Exp. section for details).

Nevertheless, the *electronic effect* has a detectable influence on the kinetics of the amide bond isomerization (Figure 2B). Indeed, in the case of 4-fluorine substitution (Ac-**5**-OMe) the barriers of the amide rotation are reduced by approx. 3 kJ mol^{-1} (rate constant enhancement by a factor ~ 4-5), when compared to both unsubstituted and 4-methylated analogues (Ac-**4**/**6**-OMe). This observation correlates well with the previously observed tendency for 4-substituted prolines: reduction of the ammonium pK_a in the amino acid by one unit resulted in approx. 1 kJ mol^{-1} reduction of the amide rotational barriers in Ac-AA-OMe models.[36]

Remarkably, the rotational barriers in all 2,4-methanoproline derivative Ac-**4**/**5**/**6**-OMe exhibit a vast increase of the amide rotation velocities, and due to this fact the direct observation of the *cis*-rotamer at the standard temperature was not possible. When compared to proline (Ac-**2**-OMe), the rate increase was about four and three orders of magnitude for *cis*→*trans* and *trans*→*cis* transitions, respectively.

Structure analysis

The unusually high *trans*-amide preferences and low amide rotation rates observed in 2,4-methanoprolines motivated us to investigate additional structural properties of the model compounds. To this end, we analyzed X-ray crystal structures of the model substances (Figure 4).[37]

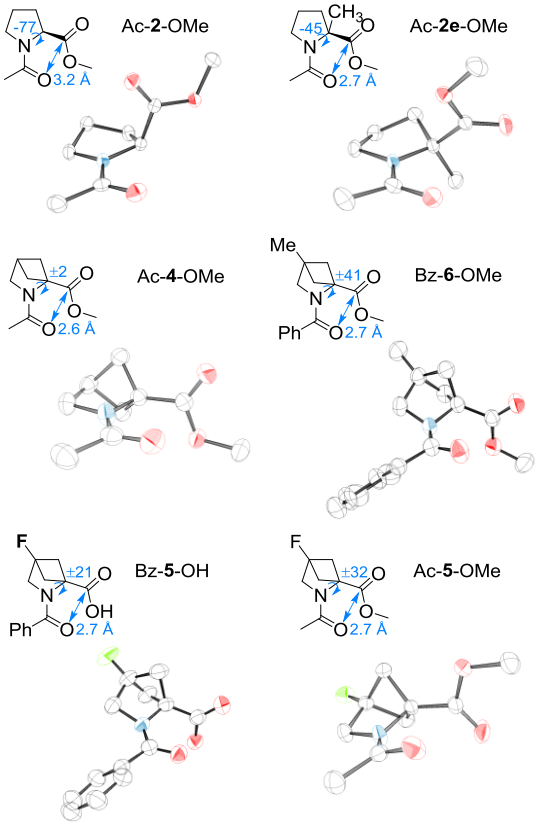


Figure 4 X-ray crystal structures for amino acid derivatives.[a]

[a] Black – carbon, blue – nitrogen, red – oxygen, green – fluorine. The ϕ-angles, and the C=O↔C=O distance of the putative n→π* interaction are highlighted in light blue. Elipsoids contours represent 50% displacement probability.

Firstly, a high *trans*-amide content should be expected for a quaternary proline analogue due to apparent steric reasons. Following this argument, an H-to-CH_3 substitution in position 2 of the proline ring (as in 2-methylproline) should potentially result in a higher *trans*-amide

dominance as compared to H-to-CH_2 substitution (as in 2,4-methanoprolines). This conclusion is however contradicted by our experimental observations, which revealed exactly the opposite tendency: the *trans*-amide in Ac-**2e**-OMe is at least 1 kJ mol^{-1} less favored than in Ac-**4**/**5**/**6**-OMe (Figure 2A). This suggests higher steric requirements for amide rotation around 2,4-methanoproline residues, which may occur due to the rigid conformation of the molecule. Similarly, extraordinary high *trans*-amide dominance has been reported in another proline analogues based on 7-azabicyclo[2.2.1]heptane skeleton, where an • -substituent was located at a bridgehead carbon atom.[38]

Indeed, unlike in proline or 2-methylproline, the C-terminal carboxymethyl group in 2,4-methanoprolines is located at the bridgehead atom, and therefore, it should potentially yield a zero ϕ-angle [C(=O)-N-C-C(=O) torsion]. This implies that the carboxymethyl group should reside in exactly the same plane as the acetyl substituent, and this placement inevitably features a steric clash between the two groups, destabilizing the amide ground states in the 2,4-methanoproline derivatives. This putative steric clash pushes the carboxymethyl group out of plane, yielding non-zero experimental ϕ-angles (±2-41 in crystal, ±11-42 in DFT modelled structures, see Table S5). The significant steric interference of the C-terminal carboxylate and the acetyl moiety may explain the observed vast reduction of the amide rotational barriers for 2,4-methanoproline derivatives (by about 15-22 kJ mol^{-1} when compared to proline).

Further analysis of the structures provides indications for a strong n→π* interaction between the carbonyl groups. For all three 2,4-methanoprolines the found N-C=O↔C=O distance was about 2.7 Å, which is remarkably lower than the value found in Ac-Pro-OMe (~ 3.2 Å)[36] or in the hexaproline crystal structures (2.9 – 3.2 Å),[39] but similar to that found in the crystal

structure of Ac-**2e**-OMe (2.7 Å) or a hexameric structure of an *exo*-pucker stabilizing proline analogue (2.7 – 2.9 Å).[15] This allows us to conclude that the n→π* donation is a prominent feature of 2,4-methanoprolines and that it is even stronger than in parent prolines. This conclusion is further supported by the fact, that the $^{acyl}O\ldots C{=}O^{carboxyl}$ angle observed in the crystal structures (99-107°) is very close to the Bürgi-Dunitz angle (107°), at which the interaction is optimal. The presence of the n→π* interaction dictated by the rigid 2,4-methanoproline scaffold can explain the large preference in favor of the *trans*-conformation observed in solution.

Finally, the absence of an *electronic effect* influence can be explained if one considers possible opposing forces, namely the n→π* donative attractive interaction and the n)(π repulsion of the carbonyl groups (Figure 5A).[40] If both interactions experience the electron withdrawing effect of the 4-fluorine substituent to the same extent, the overall effect may be cancelled out. Indeed, in the salt forms of Ac-**4/5/6**-O^-, in which the electrophilicity of the C-terminal group is the smallest and the n→π* donation should be attenuated, we observed an increase of the *trans*-amide stability for 4-fluoro-2,4-methanoproline by about -0.9 ± 0.1 kJ mol^{-1} (Figure 5B). This outcome may appear due to the repulsion of the carbonyl groups, which is suppressed by the electron withdrawing effect of the 4-fluorine substitution.

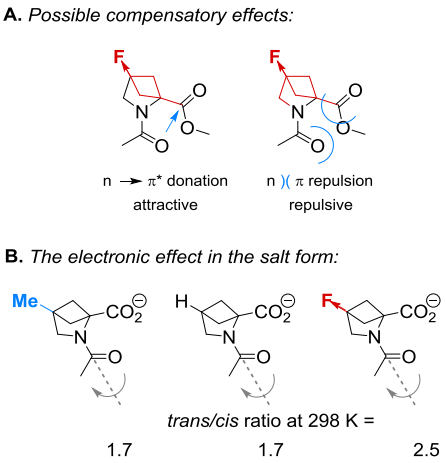


Figure 5 n →π* donation and n)(π repulsion are the two contradicting forces affecting the *trans/cis* amide equilibrium. In the salt forms of *N*-acetyl amino acids, the n →π* donation is diminished, however, the 4-fluorine *electronic effect* on repulsion force increases the *trans*-amide content.

Conclusions

In summary, we found that the 2-azabicyclo[2.1.1]hexane skeleton in 2,4-methanoproline residues creates a unique steric interference between the C- and N-side carbonyl groups placed in the same plane. We also conclude that this arrangement enables an effective n→π* donative stabilization in the *trans*-amide conformation, as seen from the structure analysis. An outcome of these steric and electronic phenomena is remarkably high *trans/cis* amide propensities in 2,4-methanoproline derivatives. The latter were characterized with the values expected for secondary rather than tertiary amides, which is a significant result for a seemingly trivial one-carbon atom addition in an amino acid structure.

A replacement of a hydrogen atom in position C-4 by a substituent does not impact the side chain conformation, and subsequently a pure *electronic effect* was followed by pK_a measurements. While the 4-methyl group does not exhibit such an effect, a 4-fluorine demonstrates a significant electron-withdrawing effect on both functional groups of the amino acid. The 4-fluorine substitution exerts a further effect onto amide rotation barriers, giving rise to a rotation rate enhancement by a factor of ~ 4-5. However, only a marginal influence onto the *trans/cis* amide preferences has been found. As a result, we conclude that the *electronic effect* of the 4-substituent influences only the kinetic behavior but not the thermodynamic stability of the *trans*-amide bond in the studied amino acid model system.

Experimental section

General notes

Solvents were purified according to standard procedures. Other starting materials were taken from commercial sources at the highest commercial quality and used without further purification. Column chromatography was performed using Kieselgel Merck 60 (230-400 mesh) as the stationary phase. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous material, unless otherwise stated. ^{1}H, ^{19}F, ^{13}C NMR spectra were recorded at 700, 500 or 400 MHz (^{1}H), 659, 471 or 376 MHz (^{19}F) and 126 or 101 MHz (^{13}C) frequencies. Chemical shifts are reported in ppm downfield from TMS (^{1}H, ^{13}C) or $CFCl_3$ (^{19}F) according to the deuterium lock referencing. The *N*-acetyl amino acid samples were measured in deuterium oxide with 150 mM $KDSO_4$, the samples were prepared as described earlier.[23] ^{13}C{^{1}H} dept45, ^{13}C^{1}H HSQC and ^{13}C^{1}H HMBC experiments were carried out for the assignment of ^{1}H and ^{13}C resonances. Mass spectra were recorded on a LCMS instrument with chemical ionization (CI) or a GCMS instrument with electron impact ionization (EI). High-resolution mass analysis (HRMS) was done using electrospray-orbitrap ionization-detection scheme. For the compounds **8**, **9**, Bz-**5**-OMe and **5**·HCl reference spectra were reported in [30].

Synthesis of 4-fluoro-2,4-methanoproline

2-Fluoroprop-2-enyl methanesulfonate (8)

40% solution of 2-fluoroprop-2-en-1-ol in Et_2O (5.0 g, 0.026 mol; obtained as described)[30], was diluted with CH_2Cl_2 (20 ml), and diisopropylethylamine (DIPEA; 6.33 ml, 0.036 mol) was added. The resulting solution was cooled to −30 ºC, and MsCl (2.42 ml, 0.031 mol) was added dropwise. The reaction mixture was allowed to warm to room temperature, and

stirred for additional 1 h. The mixture was washed with water, 10% solution of citric acid, brine, dried over Na_2SO_4, and evaporated under reduced pressure. The crude product was purified by flash column chromatography using pentane/EtOAc = 7/1 as an eluent (R*f* = 0.3) to afford the pure compound **8** as a colorless oil. ^{1}H NMR (500 MHz; $CDCl_3$; Me_4Si), δ: 3.09 (s, 3H, C*H*$_3$), 4.73 (d, $^3J_{H\text{-}F}$ = 16.0 Hz, 2H, C*H*$_2$OMs), 4.61 (dd, *J* = 46.5, 3.5 Hz, 1H, C*H*H), 4.97 (dd, *J* = 15.0, 2.0 Hz, 1H, CH*H*). ^{19}F NMR (470 MHz; $CDCl_3$; $CFCl_3$), δ: −109.0 (m).

Methyl 2-[benzoyl(2-fluoroprop-2-enyl)amino]acrylate (9)

Amino acid **7** was synthesized following the modified procedure reported previously.[30] A solution of KO*t*Bu (3.30 g, 29.3 mmol, 2.1 eq.) in THF (100 ml) was treated under argon at −78 ºC with a solution of methyl *N*-benzoyl-3-chloroalaninate **7** (3.30 g, 13.7 mmol, 1.0 eq) in THF (50 ml) followed by addition of **8** (6.30 g, 40.8 mmol, 3.0 eq). The reaction mixture was allowed to warm to room temperature, stirred for 12 h, and partitioned between CH_2Cl_2/H_2O (500/100 mL). The organic layer was separated and washed with 10% solution of citric acid, brine, dried over Na_2SO_4, and evaporated under reduced pressure. The crude product was purified by flash column chromatography (pentane/EtOAc = 7/1, R*f* = 0.3) to afford the title compound **9** as a colorless oil (2.81 g, 10.8 mmol, 79% yield). The compound was of 80% purity, and was used in the next step without additional purification. ^{1}H NMR (500 MHz; $CDCl_3$; Me_4Si), δ: 3.67 (s, 3H, C*H*$_3$), 4.73 (d, $^3J_{H\text{-}F}$ = 13.0 Hz, 2H, NC*H*$_2$), 4.63 (d, $^3J_{H\text{-}F}$ = 49.0 Hz, 1H, CF=C*H*H), 4.81 (d, $^3J_{H\text{-}F}$ = 17.0, 1H, CF=CH*H*), 5.68 (s, 1H, NC=C*H*H), 6.18 (s, 1H, NC=CH*H*), 7.34-7.45 (m, 3H, Ph), 7.50 (d, $^3J_{H\text{-}H}$ = 7.0 Hz, 2H, *o*-Ph). ^{19}F NMR (470 MHz; $CDCl_3$; $CFCl_3$), δ: −106.3 (m). *m/z* (CI): 264 (M+1).

Methyl 2-benzoyl-4-fluoro-2-azabicyclo[2.1.1]hexane-1-carboxylate (Bz-5-OMe)

Compound **9** (2.65 g, 10.0 mmol) and benzophenone (500 mg) were dissolved in degassed CH_3CN (500 ml) in a 1L flask. The solution was irradiated at 366 nm during 12 h (the reaction progress was monitored by ^{1}H NMR). The reaction mixture was concentrated, and the residue was purified by flash column chromatography (hexane/EtOAc = 3/1) to give pure Bz-**5**-OMe as a colorless oil (2.40 g, 9.1 mmol, 91% yield). R*f* = 0.6 (hexane/EtOAc = 3/1). ^{1}H NMR (500 MHz; $CDCl_3$; Me_4Si), δ: 2.34 (br s, 2H, 2 × C*H*H), 2.44 (br s, 2H, 2 × CH*H*), 3.61 (s, 2H, NCH_2), 3.81 (s, 3H, CH_3), 7.43 (t, $^3J_{H\text{-}H}$ = 7.5 Hz, 2H, Ph), 7.51 (t, $^3J_{H\text{-}H}$ = 7.5 Hz, 1H, Ph), 7.73 (d, $^3J_{H\text{-}H}$ = 7.0 Hz, 2H, Ph). ^{13}C NMR (126 MHz; $CDCl_3$; Me_4Si), δ: 47.0 (d, $^2J_{C\text{-}F}$ = 10 Hz, 2 × *C*H_2), 52.6 (s, O*C*H_3), 53.9 (d, $^2J_{C\text{-}F}$ = 28 Hz, N*C*H_2), 60.4 (d, $^3J_{C\text{-}F}$ = 23 Hz, *C*CO_2CH_3), 87.8 (d, $^1J_{C\text{-}F}$ = 265 Hz, *C*F), 127.1 (s, *C*H, Ph), 128.6 (s, *C*H, Ph), 132.1 (s, *C*H, Ph), 133.2 (s, *C*, Ph), 167.7 (d, $^4J_{C\text{-}F}$ = 11 Hz, *C*O_2CH_3), 173.5 (s, N*C*OPh). ^{19}F NMR (470 MHz; $CDCl_3$; $CFCl_3$), δ: −173.1 (s). *m/z* (CI): 264 (M+1). Anal. calcd for $C_{14}H_{14}FNO_3$: C, 63.87; H, 5.36; N, 5.32. Found: C, 63.52; H, 5.14; N, 5.67.

2-Benzoyl-4-fluoro-2-azabicyclo[2.1.1]hexane-1-carboxylic acid (Bz-5)

Compound Bz-**5**-OMe (2.20 g, 8.36 mmol) was dissolved in MeOH (15 ml), and this solution was added to 2 N NaOH (41.8 mmol, 1.60 g, 5 equiv) upon stirring. Resulting emulsion was stirred at room temperature for 10 h. The solvent was evaporated, and water (100 ml) was added. The aqueous layer was washed with CH_2Cl_2 (3x20 mL) and the organic layer was discarded. The water phase was acidified with 2 N HCl, and extracted with EtOAc (3x100 mL). The organic layers were combined, dried over Na_2SO_4 and concentrated under reduced pressure to afford the title compound Bz-**5** as a yellowish solid (1.93 g, 7.78 mmol, 93% yield). M. p. = 176-177 ºC. ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si), δ: 2.36 (s, 2H, 2 × C*H*H), 2.54 (br s, 2H, 2 × CH*H*), 3.61 (s, 2H, NCH_2), 7.43 (t, $^3J_{H\text{-}H}$ = 7.4 Hz, 2H, Ph), 7.52 (t, $^3J_{H\text{-}H}$ = 7.4 Hz, 1H, Ph), 7.70

(d, $^{3}J_{H\text{-}H}$ = 7.0 Hz, 2H, Ph). ^{13}C NMR (101 MHz; $CDCl_3$; Me_4Si), δ: 47.7 (d, $^{2}J_{C\text{-}F}$ = 21 Hz, 2 × *C*H_2), 54.6 (d, $^{2}J_{C\text{-}F}$ = 29 Hz, N*C*H_2), 61.9 (d, $^{3}J_{C\text{-}F}$ = 23 Hz, *C*CO_2H), 87.6 (d, $^{1}J_{C\text{-}F}$ = 265 Hz, *C*F), 128.5 (s, *C*H, Ph), 128.8 (s, *C*H, Ph), 132.3 (s, *C*H, Ph), 133.1 (s, *C*, Ph), 170.5 (d, $^{4}J_{C\text{-}F}$ = 11 Hz, *C*O_2H), 174.0 (s, N*C*OPh). ^{19}F NMR (376 MHz; $CDCl_3$; $CFCl_3$), δ: −170.4 (s). *m/z* (CI): 250 (M+1). Anal. calcd for $C_{13}H_{12}FNO_3$: C, 62.65; H, 4.85; N, 5.62. Found: C, 62.39; H, 4.55; N, 5.83.

1-Carboxy-4-fluoro-2-azabicyclo[2.1.1]hexan-2-ium chloride (5·HCl)

Bz-**5** (0.6 g, 2.4 mmol) was dissolved in 6 N HCl (30 ml) upon stirring. The mixture was refluxed for 4 h. The solvent was removed under reduced pressure, and the crude material was dissolved in water and hot filtered with activated charcoal. The filtrate was freeze-dried to afford **5**·HCl as a grey solid (0.35 g, 1.9 mmol, 80 % yield). M. p. > 220 ºC. ^{1}H NMR (700 MHz; D_2O; Me_4Si), δ: 2.34 (dt, *J* = 5.6 and 1.5 Hz, 2H, 2 × C*H*H), 2.63 (br m, 2H, 2 × CH*H*), 3.46 (s, 2H, NCH_2). ^{13}C NMR (176 MHz; D_2O; Me_4Si), δ: 44.7 (d, $^{2}J_{C\text{-}F}$ = 21 Hz, 2 × *C*H_2), 46.2 (d, $^{2}J_{C\text{-}F}$ = 30 Hz, N*C*H_2), 60.7 (d, $^{3}J_{C\text{-}F}$ = 22 Hz, N*C*COOH), 87.6 (d, $^{1}J_{C\text{-}F}$ = 265 Hz, *C*F), 168.3 (d, $^{4}J_{C\text{-}F}$ = 10 Hz, *C*OOH). ^{19}F NMR (659 MHz; D_2O; $CFCl_3$), δ: −170.5 (s). *m/z* (CI): 146 (M+1). Anal. calcd for $C_6H_9ClFNO_2$: C, 39.69; H, 5.00; N, 7.71. Found: C, 39.87; H, 5.11; N, 7.45.

Synthesis of 4-methyl-2,4-methanoproline

Methyl 2-(*N*-(2-methylallyl)benzamido)acrylate (11)

Method A: A solution of KO*t*Bu (3.31 g, 29.3 mmol, 2.1 eq.) in THF (100 ml) was treated under argon at -78 ºC with a solution of methyl *N*-benzoyl-3-chloroalaninate **7** (3.30 g, 13.7 mmol, 1.0 eq) in THF (50 ml) followed by addition of **10** (5.50 g, 40.8 mmol, 3.0 eq). The reaction mixture was allowed to warm to room temperature, stirred for 12 h, and partitioned

between CH_2Cl_2/H_2O (500/100 mL). The organic layer was separated, washed with 10% solution of citric acid, brine, dried over Na_2SO_4, and evaporated under reduced pressure. The crude product was purified by flash column chromatography (pentane/EtOAc = 3/1, R*f* = 0.6) to afford the title compound **11** as a colorless oil (2.70 g, 10.8 mmol, 76% yield). Method B: A three necked, 2 L round-bottomed flask was fitted with a mechanical stirrer and charged with methyl pyruvate **12** (102.0 g, 1.00 mol) in 1L of toluene; then 2-methylallylamine **13** (71.0 g, 1.0 mol) was added and the mixture was stirred for 4 hours at room temperature under argon. After stirring, the reaction mixture was poured into water and the organic layer was separated, dried over Na_2SO_4 and filtered. The filtrate was cooled to −10-0ºC and triethylamine (101.0 g, 1.00 mol) was added followed by benzoyl chloride (112.0 g , 0.80 mol) while the temperature was maintained. The resulting mixture was allowed to warm to room temperature and stirred overnight. The precipitate of triethyamine hydrochloride was filtered and washed with toluene. The filtrate was concentrated under reduced pressure and purified by flash chromatography (pentane/EtOAc = 3/1, R*f* = 0.6) to afford the title compound **11** as a yellow oil (155.4 g, 0.60 mol, 60% yield). ^{1}H NMR (400 MHz; methanol-d_4; Me_4Si), δ: 1.72 (s, 3H, C*H*$_3$), 3.51 (s, 3H, OC*H*$_3$), 4.24 (s, 2H, C*H*$_2$), 4.89 (br s, 2H, CH_3C=C*HH*), 5.64 (s, 1H, NC=C*H*H), 6.01 (s, 1H, NC=CH*H*), 7.13-7-51 (5H, m, Ph). ^{13}C NMR (101 MHz, methanol-d_4; Me_4Si), δ: 20.7 (s), 53.0 (s), 55.9 (broad s), 114.1 (s), 122.9 (broad s), 129.0 (s), 129.3 (s), 131.6 (s), 137.1 (s), 141.7 (s), 142.2 (s), 165.6 (s), 173.4 (broad s). *m/z* (CI): 260 (M+1). Anal. calcd for $C_{15}H_{17}NO_3$: C, 69.48; H, 6.61; N, 5.40. Found: C, 69.11; H, 6.32; N, 5.71.

Methyl 2-benzoyl-4-methyl-2-azabicyclo[2.1.1]hexane-1-carboxylate (Bz-6-OMe)

The compound **11** (11.8 g, 0.045 mol) was dissolved in distilled acetonitrile (1 L) and acetophenone (2.5 g, 0.021 mol) was added. The reaction was irradiated at 366 nm, and the

reaction progress was monitored by NMR. After the reaction completeness, the mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography using pentane/EtOAc = 2/1 as an eluent. R*f* = 0.4.The product Bz-**6**-OMe as white solid (11.6 g, 0.042 mol, 94% yield). M. p. = 100-101 ºC. ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si), δ: 1.28 (s, 3H), 1.84 (br s, 2H, 2 × C*H*H), 1.98 (br s, 2H, 2 × CH*H*), 3.37 (s, 2H, NCH_2), 3.79 (s, 3H, CH_3), 7.40 (t, $^{3}J_{H\text{-}H}$ = 7.6 Hz, 2H, Ph), 7.51 (t, $^{3}J_{H\text{-}H}$ = 7.3 Hz, 1H, Ph), 7.75 (d, $^{3}J_{H\text{-}H}$ = 7.0 Hz, 2H, *o*-Ph). ^{13}C NMR (101 MHz; $CDCl_3$; Me_4Si), δ: 16.5 (*C*H_3), 43.7 (s, *C*CH_3), 46.3 (2 × *C*H_2), 52.2 (s, O*C*H_3), 59.8 (s, N*C*H_2) 68.1 (*C*CO_2CH_3), 128.3 (s, *C*H, Ph), 128.5 (s, *C*H, Ph), 131.4 (s, *C*H, Ph), 134.4 (s, *C*, Ph), 169.0 (s,CO_2Me), 173.5 (s, N*C*OPh). *m/z* (CI): 260 (M+1). Anal. calcd for $C_{15}H_{17}NO_3$: C, 69.48; H, 6.61; N, 5.40. Found: C, 69.23; H, 6.84; N, 5.12.

1-Carboxy-4-ethyl-2-azabicyclo[2.1.1]hexan-2-ium chloride (6·HCl)

A suspension of Bz-**5**-OMe (0.73 mol, 200 g) in 6N HCl (1L) was brought to reflux for 10 hours, then cooled to 0 ºC, the resulting precipitate (benzoic acid) was filtered, and washed with 200 ml of cold water. The filtrate was concentrated under reduced pressure. The resulting solid residue was triturated in dry acetone to afford the title product **6**·HCl as a white solid (124.3 g, 0.7 mol, 97% yield). M. p..> 250 ºC. ^{1}H NMR (700 MHz; D_2O; Me_4Si), δ: 1.26 (s, 3H, CH_3), 1.82 (dd, *J* = 5.9 and 2.4 Hz, 2H, 2×C*H*H), 2.18 (d, *J* = 6.0 Hz, 2H, 2× CH*H*), 3.23 (s, 2H, NCH_2). ^{13}C NMR (176 MHz; D_2O; Me_4Si), δ: 14.4 (s, *C*H_3), 44.1 (s, 2×*C*H_2), 45.5 (s, *C*CH_3), 52.6 (s, N*C*H_2), 68.9 (s, N*C*COOH), 168.9 (s, *C*OOH). *m/z* (CI): 142 (M+1). Anal. calcd for $C_7H_{12}ClNO_2$: C, 47.33; H, 6.81; N, 7.89. Found: C, 47.68; H, 6.52; N, 7.51.

Synthesis of 2,4-methanoproline

1-Carboxy-2-azabicyclo[2.1.1]hexan-2-ium chloride (4·HCl)

N-Boc amino acid Boc-**4** (91 mg, 0.4 mmol) was stirred in 3 M hydrogen chloride in dioxane solution (4 mL) for 14 hours. The solvent was removed under reduced pressure, the residue was taken up in water (5 mL) and freeze-dried to afford title compound **4**·HCl as a white powder (60 mg, 0.37 mmol, 92%). M. p. > 200 °C. ^{1}H NMR (700 MHz; D_2O; Me_4Si), δ: 1.73 (dd, *J* = 6.0 and 2.2 Hz, 2H, 2×C*H*H), 2.37 (br m, 2H, 2× CH*H*), 2.89 (t, *J* = 3.3 Hz, 1H, C*H*), 3.40 (s, 2H, NCH_2). The 1H NMR spectrum is consistent with the literature reported one (in methanol-d_4).[42] ^{13}C NMR (176 MHz; D_2O; Me_4Si), δ: 36.8 (s, *C*H), 40.2 (s, 2×*C*H_2), 49.4 (s, N*C*H_2), 71.6 (s, N*C*COOH), 169.2 (s, *C*OOH). *m/z* (CI): 128 (M+1). Anal. calcd for $C_6H_{10}ClNO_2$: C, 44.05; H, 6.16; N, 8.56. Found: C, 44.12; H, 5.99; N, 8.40.

Synthesis of the model compounds

2-Acetyl-2-azabicyclo[2.1.1]hexane-1-carboxylic acid (Ac-4)

4·HCl (50 mg, 0.31 mmol) was mixed with acetic anhydride (0.8 mL, 8.5 mmol, 22 equiv.) and triethylamine (0.2 mL, 1.4 mmol, 3.7 equiv.) in tetrahydrofuran – dichloromethane mixture (7 mL / 10 mL). The mixture was stirred at the room temperature until clear solution was obtained (14 hours). Solvents were removed under reduced pressure, the residue was dissolved in water (2 mL) in order to quench residual amounts of the anhydride. Resulting solution was freeze-dried, the residue was taken up in minimal amount of water (0.3 mL) and eluted through a short cation exchange resin (Dowex 50WX8, 50-100 mesh, volume 2 mL) by deionized water. Acidic fractions were collected and freeze-dried. Ac-**4** was afforded as a white powder (52 mg, 3.1 mmol, quant.). HRMS, *m/z* (ESI): calculated for $C_8H_{12}NO_3^+$ $[M+H]^+$ 170.0812; found 170.0809. ^{1}H NMR (700 MHz; D_2O+$KDSO_4$; Me_4Si), δ: 3.55 (s, 2H, δ-CH_2), 2.78 (t, *J* = 3.4 Hz, 1H, γ-CH), 2.12 (m, 2H, β-CH), 1.98 (s, 3H, CH_3C=O), 1.62 (dd, *J* = 4.8, 2.0 Hz, 2H, β-CH). ^{13}C

NMR (126 MHz; D_2O+$KDSO_4$; Me_4Si), δ: 173.4 (s, CO_2H), 172.7 (s, O=C-N), 70.1 (s, α-C), 52.2 (s, δ-CH_2), 42.1 (s, β-CH_2), 34.0 (s, γ-CH), 20.7 (s, *C*H_3C=O).

2-Acetyl-4-fluoro-2-azabicyclo[2.1.1]hexane-1-carboxylic acid (Ac-5)

Compound was synthesized in analogous procedure starting from **5**·HCl (59 mg, 0.32 mmol) using dichloromethane as a solvent. The target compound Ac-**5** was obtained as a yellowish powder (55 mg, 0.29 mmol, 90% yield). HRMS, *m/z* (ESI): calculated for $C_8H_{11}FNO_3^+$ $[M+H]^+$ 188.0717; found 188.0715. ^{1}H NMR (700 MHz; D_2O+$KDSO_4$; Me_4Si), δ: 3.65 (s, 2H, δ-CH_2), 2.38 (m, 2H, β-CH), 2.18 (m, 2H, β-CH), 1.94 (s, 3H, CH_3C=O). ^{13}C NMR (126 MHz; D_2O+$KDSO_4$; Me_4Si), δ: 173.5 (s, O=C-N), 171.1 (d, *J* = 11 Hz, CO_2H), 86.8 (d, *J* = 263 Hz, γ-CF), 60.5 (d, *J* = 25 Hz, α-C), 50.3 (d, *J* = 28 Hz, δ-CH_2), 47.1 (d, *J* = 20 Hz, β-CH_2), 20.5 (s, *C*H_3C=O). ^{19}F NMR (659 MHz; D_2O+$KDSO_4$; Me_4Si), δ: −173.3 (s).

2-Acetyl-4-methyl-2-azabicyclo[2.1.1]hexane-1-carboxylic acid (Ac-6)

6·HCl (0.3 g, 1.7 mmol) was stirred with acetic anhydride (0.5 mL, 5.3 mmol, 3 equiv.) and triethylamine (0.4 mL, 2.9 mmol, 1.7 equiv.) in dichloromethane (5 mL) until a clear solution was obtained (1 hour). The solvent was removed under reduced pressure, the residue was taken up in water (5 mL) and freeze-dried. Resulting crude material was taken up in water (3 mL) and this solution was passed through a cation exchange resin column (3.5 mL), eluted with pure water. Acidic fractions were collected and freeze-dried. Ac-**6** was obtained as a white powder (0.30 g, 1.6 mmol, 97% yield). HRMS, *m/z* (ESI): calculated for $C_9H_{14}NO_3^+$ $[M+H]^+$ 184.0968; found 184.0965. ^{1}H NMR (700 MHz; D_2O+$KDSO_4$; Me_4Si), δ: 3.38 (s, 2H, δ-CH_2), 1.95 (s, 3H, CH_3C=O), 1.89 (d, *J* = 4.6 Hz, 2H, β-CH), 1.67 (dd, *J* = 4.7, 1.9 Hz, 2H, β-CH), 1.21 (s, 3H, γ'-CH_3). ^{13}C NMR (126 MHz; D_2O+$KDSO_4$; Me_4Si), δ: 173.0 (s, O=C-N), 172.8 (s,

CO_2H), 68.0 (s, α-C), 56.4 (s, δ-CH_2), 46.4 (s, β-CH_2), 42.1 (s, γ-C), 20.6 (s, *C*H_3C=O), 15.1 (s, γ'-CH_3).

Methyl 2-acetyl-2-azabicyclo[2.1.1]hexane-1-carboxylate (Ac-4-OMe)

Ac-**4** (15 mg, 0.09 mmol) was dissolved in methanol (3 mL), and trimethylsilyl chloride (0.04 mL, 0.3 mmol, 3.6 equiv.) was added in order to acidify the solution. The mixture was stirred at the room temperature for 22 hours, the solvent was removed under reduced pressure, and resulting crude material was purified on a silica gel column using ethyl acetate – methanol (19:1) mixture as an eluent (R*f* = 0.6) to yield Ac-**4**-OMe as a colorless liquid which later crystalized (14 mg, 0.08 mmol, 86% yield). HRMS, *m/z* (ESI): calculated for $C_9H_{14}NO_3^+$ $[M+H]^+$ 184.0968; found 184.0966. M. p. = 82-84 ºC. ^{1}H NMR (700 MHz; D_2O; Me_4Si), δ: 3.65 (s, 3H, CH_3O), 3.53 (s, 2H, δ-CH_2), 2.77 (br m, 1H, γ-CH), 2.09 and 1.61 (two m, 4H, β-CH_2), 1.94 (s, 3H, CH_3C=O). ^{13}C NMR (151 MHz; D_2O; Me_4Si), δ: 173.0 (s, O=C-N), 171.5 (s, CO_2Me), 69.5 (s, α-C), 52.7 (s, CH_3O), 51.8 (s, δ-CH_2), 42.0 (s, β-CH_2), 34.2 (s, γ-CH), 20.6 (s, CH_3C=O).

Methyl 2-acetyl-4-fluoro-2-azabicyclo[2.1.1]hexane-1-carboxylate (Ac-5-OMe)

Compound was synthesized in analogous procedure starting from Ac-**5** (16 mg, 0.09 mmol). The crude product was purified on a silica gel column using ethyl acetate – methanol (19:1) mixture as an eluent (R*f* = 0.8). Compound Ac-**5**-OMe was obtained as yellowish crystals (15 mg, 0.07 mmol, 87%). HRMS, *m/z* (ESI): calculated for $C_9H_{13}FNO_3^+$ $[M+H]^+$ 202.0874; found 202.0874. M. p. = 96-98 ºC. ^{1}H NMR (700 MHz; D_2O; Me_4Si), δ: 3.71 (s, 3H, CH_3O), 3.68 (s, 2H, δ-CH_2), 2.41 and 2.22 (two br m, 4H, β-CH_2), 1.96 (s, 3H, CH_3C=O). ^{13}C NMR (151 MHz; D_2O; Me_4Si), δ: 173.1 (s, O=C-N), 169.8 (d, *J* = 11 Hz, CO_2Me), 87.0 (d, *J* = 262 Hz, γ-

CF), 60.0 (d, *J* = 24 Hz, α-C), 53.1 (s, CH_3O), 50.0 (d, *J* = 29 Hz, δ-CH_2), 46.9 (d, *J* = 21 Hz, β-CH_2), 20.4 (s, CH_3C=O). ^{19}F NMR (659 MHz; D_2O; Me_4Si), δ: −172.5 (s).

Methyl 2-acetyl-4-methyl-2-azabicyclo[2.1.1]hexane-1-carboxylate (Ac-6-OMe)

Ac-**6** (0.30 g, 1.6 mmol) was dissolved in methanol (8 mL), trimethylsilyl chloride (0.3 mL, 2.4 mmol, 1.4 equiv) was added, and resulting acidic mixture was stirred at the room temperature for 14 hours. Methanol was removed under reduced pressure. Crude material was purified on a silica gel column using ethyl acetate – methanol (20:1) mixture as an eluent (R*f* = 0.5). Analytically pure Ac-**6**-OMe was obtained as a clear oil (96 mg, 0.5 mmol, 30% yield). HRMS, *m/z* (ESI): calculated for $C_{10}H_{16}NO_3^+$ $[M+H]^+$ 198.1125; found 198.1122. ^1H NMR (700 MHz; D_2O; Me_4Si), δ: 3.68 (s, 3H, CH_3O), 3.40 (s, 2H, δ-CH_2), 1.95 (s, 3H, CH_3C=O), 1.89 (d, *J* = 5.0 Hz, 2H, β-CH), 1.69 (dd, *J* = 4.8 and 1.8 Hz, 2H, β-CH), 1.23 (s, 3H, γ'-CH_3). ^{13}C NMR (151 MHz; D_2O; Me_4Si), δ:172.7 (s, O=C-N), 171.6 (s, CO_2Me), 67.4 (s, α-C), 56.0 (s, δ-CH_2), 52.7 (s, CH_3O), 46.2 (s, β-CH_2), 42.3 (s, γ-C), 20.5 (s, *C*H_3C=O), 15.1 (s, γ'-CH_3).

(*S*)-1-Acetyl-2-methylpyrrolidine-2-carboxylic acid (Ac-2e)

2-methylproline (228 mg, 1.8 mmol) was stirred with acetic anhydride (0.6 mL, 6.4 mmol, 3.6 equiv.) and triethylamine (0.3 mL, 2.2 mmol, 1.2 equiv.) in dichloromethane (4 mL) until a clear solution was obtained (4 hours). The solvent was removed under reduced pressure, the residue was taken up in water (4 mL) and freeze-dried. Resulting material was dissolved in water (1.5 mL) and passed through a short cation exchange column (3 mL) using deionized water as an eluent. Acidic fractions were collected and freeze-dried. Ac-**2e** was obtained as a white powder (278 mg, 1.6 mmol, 92% yield). HRMS, *m/z* (ESI): calculated for $C_8H_{14}NO_3^+$ $[M+H]^+$ 172.0968; found 172.0964. $[\alpha]_D^{25} = -145$ ($CHCl_3$, c = 1.0) or −22 (MeOH, c = 1.1). Higher

optical rotation in chloroform may occur due to γ-turn formation in unipolar media. ^{1}H NMR (700 MHz; D_2O+$KDSO_4$; Me_4Si), δ: 3.72 and 3.67 (two m, 2H, δ-CH_2), 2.21 and 2.02 (two m, 2H, β-CH_2), 2.07 (s, 3H, CH_3C=O), 2.05 (M, 2H, γ-CH_2), 1.49 (s, 3H, α'-CH_3). ^{13}C NMR (126 MHz; D_2O+$KDSO_4$; Me_4Si), δ: 178.1 (s, CO_2H), 172.0 (O=C-N), 65.9 (s, α-C), 49.2 (s, N-CH_2), 38.5 (s, β-CH_2), 23.3 (s, γ-CH_2), 21.9 (s, *C*H_3C=O), 20.1 (α'-CH_3).

Methyl (*S*)-1-acetyl-2-methylpyrrolidine-2-carboxylate (Ac-2e-OMe)

Ac-**2e** (71 mg, 0.41 mmol) was dissolved in methanol (2 mL) and trimethylsilyl chloride (0.1 mL, 0.86 mmol, 2.1 equiv.) was added in order to acidify the mixture. This was stirred at the room temperature for 22 hours. The solvent was removed under reduced pressure, and crude material was purified by silica gel column chromatography using ethyl acetate – methanol (19:1) mixture as an eluent (R*f* = 0.5). Pure Ac-**2e**-OMe was obtained as a clear oil which later crystallized (53 mg, 0.29 mmol, 69% yield). HRMS, *m/z* (ESI): calculated for $C_9H_{16}NO_3^+$ $[M+H]^+$ 186.1125; found 186.1122. M. p. = 82-85 ºC. $[\alpha]_D^{25} = -17$ ($CHCl_3$, c = 1.0). ^{1}H NMR (700 MHz; D_2O; Me_4Si), δ: 3.66 (s, 3H, CH_3O), 3.65 and 3.60 (two m, 2H, δ-CH_2), 2.10 and 1.92 (two m, 2H, β-CH_2), 2.10 (s, 3H, CH_3C=O), 1.98 (m, 2H, γ-CH_2), 1.42 (s, 3H, α'-CH_3). ^{13}C NMR (176 MHz; D_2O; Me_4Si), δ: 176.9 (s, CO_2Me), 172.1 (s, O=C-N), 66.1 (s, α-C), 53.0 (s, CH_3O), 49.2 (s, δ-CH_2), 38.4 (s, β-CH_2), 23.3 (s, γ-CH_2), 21.9 (s, *C*H_3C=O), 20.2 (s, α'-CH_3).

Physical chemistry of the amino acids

pK_a measurements

The pK_a determination procedure is similar to reported earlier.[23] NMR measurements were performed on aqueous samples titrated to different pH values. A sample contained 2-3 mM of an analyte, 8 mM potassium phosphate, 0.05 mM sodium 3-(trimethylsilyl)propane-1-

sulfonate (TPS, internal ^{1}H standard), 1/11 protium-deuterium ratio was established by addition of deuterium oxide (for ^{2}H lock). ^{1}H NMR measurements were performed in WATERGATE W5 pulse sequence at 700 MHz resonance frequency. ^{19}F NMR spectra were recorded in single-pulse experiments without decoupling at 659 MHz frequency. The measurements were conducted at 298 K. Various chemical shift values were plotted against pH, fitted to a Boltzmann fit, and 1st order derivative indicated the pK_a value as extremum points.

By using this procedure it was possible to identify pK_a values of the amino groups in free amino acids. For the *N*-acetyl amino acids, pK_a can be measured for *N*-acetyl proline (Ac-**2**) and *N*-acetyl 2-methylproline (Ac-**2e**) in both rotameric forms. For instance, in Ac-**2e** the pK_a of the s-*trans* and s-*cis* forms was 4.06 and 2.98, respectively. Thus, the s-*cis* form is 1.08 units more acidic, and this value can be also obtained by analysis of the rotameric populations in the acidic (pH 1.4, with $KDSO_4$) and salt (phosphate buffer, pH 7.0) forms of this compound according to eqn. 1. For the salt form the $K_{trans/cis}$ value was identified as 2.56±0.09, and for the acid form this was 28.3±1.0. That corresponds to ΔpK_a = 1.04, which is close to the actual value 1.08 within the accuracy of the pK_a measurements (±0.05).

$$pKa(s-trans) - pKa(s-cis) = \Delta pKa = \frac{K_{trans/cis}(acid)}{K_{trans/cis}(salt)} \qquad (1)$$

For Ac-**4**/**5**/**6** the *trans/cis* ratio was also increasing when going to lower pH, however, the s-*cis* resonances broadened and disappeared from detection at pH ≤ 3 in both ^{1}H and ^{19}F (for Ac-**5**) spectra. The pK_a values were only determined for the s-*trans* rotameric form. Spectra from the titration series are shown on Fig. S1-S10 in the Supporting Information. The data is summarized in Tab. S1-S2.

Equilibrium *trans/cis* ratios

Salt samples of Ac-**2e**/**4**/**5**/**6** were prepared in aqueous medium by addition of potassium hydroxide to establish pH 7.0, followed by lyophilization, lyophylization from deuterium oxide, and finally the samples were dissolved in deuterium oxide for measurements. The samples contained 50 mM of *N*-acetyl amino acids and 70 mM of potassium phosphate. For measurements of the esters Ac-**2**/**2e**/**4**/**5**/**6**-OMe, analytes were dissolved in potassium phosphate buffer in deuterium oxide. The potassium phosphate buffer was first prepared in aqueous medium (pH 7.0), and subsequently lyophilized from deuterium oxide, and dissolved in deuterium oxide. The samples contained 50 mM of analytes and 70 mM of phosphate. Deuteromethanol samples were prepared by dissolving Ac-**2**/**2e**/**4**/**5**/**6**-OMe in methanol-d_4. The samples contained 50 mM of analytes.

^{1}H and ^{19}F NMR spectra were recorded at 700 and 659 MHz frequencies in 90-degree pulse experiment. The 90-deg pulse was calibrated prior the measurements. For ester equilibrium populations at lower temperatures the ^{1}H NMR spectra were recorded with ^{13}C decoupling during acquisition (inverse gated decoupling). The spectra were acquired either in single-scan or in multiscan mode with long recycling delay in order to ensure complete pre-relaxation of the analyzed resonances. The spectra were baseline corrected and integrated delivering the *trans/cis* ratios. The ratios were also obtained in reference 2D EXSY spectra (*vide infra*). In addition, the EXSY spectra were used for unambiguous assignment of the rotameric forms (exchange signals), and for the assignment of the s-*trans* rotamers (NOE: CH_3(acetyl) $\leftrightarrow$ δ-CH_2).

Variable temperature unit was calibrated by conventional acidified methanol sample referencing.[42] For the salt samples the spectra were recorded at 298 K, and the rotameric ratio is given in Tab. S1. For the ester samples the *trans/cis* ratio was determined in buffered deuterium

oxide at 298 K for Ac-**2**-OMe (4.95±0.05) and Ac-**2e**-OMe (39±1). For Ac-**4**/**5**/**6**-OMe this was not possible, and we performed a search for the s-*cis* rotameric form at lower temperatures. As exemplified at Fig. S11, the s-*cis* rotameric resonance of methoxyl group in Ac-**6**-OMe sharpens, and ^{13}C decoupling is necessary for cleaning it from the ^{13}C satellite of the dominant s-*trans* resonance. The measurements were thus performed at 276 K in buffered deuterium oxide samples using $^{1}H\{^{13}C\}$ or ^{19}F NMR detection. For the deuteromethanol samples, where the *trans*-*cis* rotation is generally faster, the measurements were performed at 262 K. For Ac-**5**-OMe the low temperature ^{19}F spectrum delivered very well separated resonances as shown on Fig. S12. The data is summarized in Tab. S3.

The amide equilibrium for Ac-**2a**-OMe and Ac-**2b**-OMe in aqueous medium have been characterized earlier.[12] For 4-methylprolines experimental *trans/cis* values were re-evaluated. The $K_{trans/cis}$ values for Ac-**2c**-OMe and Ac-**2d**-OMe were re-evaluated in aqueous solutions at 298 K. The found values were 4.67±0.14 and 8.05±0.13, respectively. The values were in parts reported in [15]. The data is shown on Fig. 3.

Kinetics of the amide isomerization

The measurements were performed in standard z-cross-relaxation experiments (EXSY/NOESY with gradients). Mixing time was inset 3 or 5 ms for referencing and variable mixing times for exchange detection. The recycling delay was $\geq 3 \cdot T_1$ for the analyzed resonances, as estimated from inversion recovery experiments. The 2D spectra were baseline corrected and integrated. Exchange rate matrices were calculated using EXSYcalc software (Mestrec). The activation energy values were calculated using Eyring equation (eqn. 2). ^{19}F cross-relaxation spectra are exemplified on Fig. S14 and S16. For **2e**, **4** and **6** derivatives

different separated resonances were analyzed in ^{1}H spectra (examples are at Fig. S13 and S15). Obtained values are summarized in Tab. S4.

$$E^{\neq} = RT(-\ln\frac{k_{\exp}}{T} + 23.76) \qquad (2)$$

Structure analysis

X-ray diffraction data was collected at 150 K. DFT molecular modelling was performed in using the B88-PW91 GGA functional with DZVP basis set. The results are summarized in Tab S5.

Associated Content

The supporting information includes Tables S1-S5 and Figures S1-S16 from physicochemical NMR interrogations, copies of the NMR spectra for substances (PDF), and crystallographic information (CIF). The supporting information is available at http://pubs.acs.org/

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