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α-Unsaturated 3-Amino-1-carboxymethyl-β-lactams as Bacterial PBP Inhibitors: Synthesis and Biochemical Assessment

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Abstract: Innovative monocyclic β-lactam entities create opportunities in the battle against resistant bacteria because of their PBP acylation potential, intrinsically high β-lactamase stability and compact scaffold. α-Benzylidene-substituted 3-amino-1-carboxymethyl-β-lactams were recently shown to be potent PBP inhibitors and constitute eligible anchor points for synthetic elaboration of the chemical space around the central β-lactam ring. The present study discloses a 12-step synthesis of ten α-arylmethylidenecarboxylates using a microwave-assisted Wittig olefination as the crucial reaction step. The library was designed aiming at enhanced β-lactam electrophilicity and extended electron flow after enzymatic attack. Additionally, increased β-lactamase stability and intermolecular target interaction were envisioned by tackling both the substitution pattern of the aromatic ring and the β-lactam C4-position. The significance of α-unsaturation was validated and the R39/PBP3 inhibitory potency shown to be augmented the most through decoration of the aromatic ring with electron-withdrawing groups. Furthermore, ring cleavage by representative β-lactamases was ruled out, providing new insights in the SAR landscape of monocyclic β-lactams as eligible PBP or β-lactamase inhibitors.

Introduction

Antibiotic resistance has led to a global health crisis, which requires joint action to avoid one of the most valuable scientific milestones of the twentieth century from being eroded in the twenty-first. Ninety years ago, Alexander Fleming serendipitously discovered benzylpenicillin, a substance produced by moulds that is able to kill bacteria.[1] This finding, followed by the discovery and development of a series of analogous antimicrobial drugs, introduced a whole new era in the field of modern medicine.[2] β-Lactam antibiotics soon became the drugs of choice for the treatment of bacterial infections due to their unparalleled clinical safety, effectiveness and unique chemotherapeutic properties. Their mode of action relies on the effective irreversible inhibition of the last step of the bacterial cell wall synthesis. A major component thereof concerns peptidoglycan, a macromolecule consisting of glycan strands cross-linked by peptides. The combination of essential and unique renders peptidoglycan a prime target for antibacterial agents. The highly strained and thus reactive β-lactams irreversibly inhibit the penicillin-binding proteins or PBPs, responsible for peptide cross-linking, by acylating the catalytic serine residue. The main driving force responsible for the binding of the β-lactam into the active site is the functional mimicry of the enzyme's native substrate, the C-terminal D-alanyl-D-alanine moiety of the precursor stem peptide found in the nascent peptidoglycan layer.[3] PBP inhibition leads to a significant weakening of the bacterial cell wall and ultimately provokes cell lysis.[4]

The propagation of drug-resistant bacterial strains, however, jeopardises the effectiveness of antibiotic treatments.[5,6] Current serious threats include drug-resistant forms of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species, nicknamed together as the 'ESKAPE' pathogens, which, among other superbugs, cause 700,000 deaths a year.[7] If the resistance phenomenon continues to expand at the same pace, a catastrophic scenario is impending, in which the burden of resistance-related deaths could rise to 10 million people a year by 2050.[2,8] Still, while doom scenarios are being forecast, the steady stream of antibiotics and classes being launched on the market since the "golden era" of antibiotic discovery (1940s–1970s) has only declined.[2,8,9] In order to avoid healthcare returning to primitive conditions, countering

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antibiotic resistance will thus require continuous investments in a functioning R&D pipeline.

While some pathogens evade β-lactam action by the production of β-lactamases, PBP-like enzymes that hydrolyse the β-lactam ring, others recruit or (over)express low-affinity PBPs whose active site cavity is severely distorted (e.g., PBP5fm of *Enterococcus faecium*).[10–12] In view of overcoming the intrinsically low acylation susceptibility of the latter modified PBPs, our group concentrates on the design of β-lactams that are more restricted in volume than classic bicyclic penicillins **1** and cephalosporins **2**, but still retain their acylation potential: monocyclic β-lactams (Figure 1). Our recent preliminary work focused on the in silico design of new 3-amino-1-carboxymethyl-β-lactams as direct D-Ala-D-Ala mimetics.[13] Taking into account the recently reviewed knowledge on monocyclic β-lactam antibacterials,[14] and combining the structural properties of aztreonam **3** (the only FDA-approved monobactam), the nocardicins **4** (natural monocyclic β-lactams) and late-generation cephalosporins **2** (e.g., ceftobiprole), a new pharmacophore **5** was designed and studied (Figure 2). It consisted of *i.* a 4-unsubstituted 3-amino-1-carboxymethyl-β-lactam nucleus, mimicking the natural PBP substrate, enabling crucial interactions with the active site and securing the limited size of the core skeleton—and hence its potential to enter an active-site-distorted target; *ii.* an established 2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetamido substituent linked to the β-lactam C3-position;[14] and finally, *iii.* a side chain 'R' as a variable item that was examined using a hierarchical structure-based virtual screening process based on the crystal structure of PBP5fm of *E. faecium*. Overall, most side chains 'R' were found to embed a lipophilic linker and a terminal hydrogen bond-donating or -accepting functionality ('HBD/HBA'), enabling additional non-covalent interactions with the enzyme.

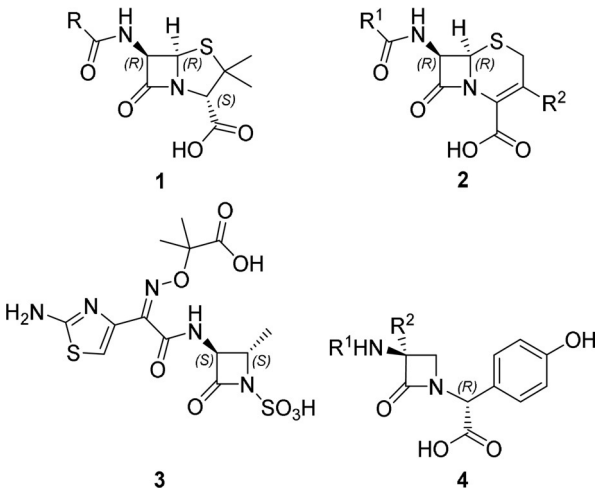


Figure 1. β-Lactam antibiotics. Penicillins **1** (benzylpenicillin **1A**: R = Bn), cephalosporins **2**, aztreonam **3**, nocardicins **4**.

Keeping the latter in mind, a ten-step synthetic pathway towards a broad, model library of optically pure target compounds **6** was developed.[13] A counter-labelling assay of this first series demonstrated the promising *E. coli* PBP3 inhibitory potential of benzylidene derivative **6A$_a$** (residual activity of 28%). Slight inhibition of two other PBPs (PBP5fm of resistant *E. faecium* and *Actinomadura* R39) and a high β-lactamase stability with even weak inhibition of class C β-lactamase P99, prompted us to promote this derivative to become our first hit. Albeit not the only requisite for activity, the presumed higher chemical reactivity and increased electron flow through the additional double bond and the aromatic ring were suggested to elevate the inhibitory activity of compound **6A$_a$** compared to other, and even very close derivatives lacking this unsaturated moiety. The electron flow might allow resonance stabilisation of the negative charge on the β-lactam nitrogen atom formed after acylation of the catalytic serine residue. Similar observations are made upon comparison of Δ^3-cephalosporins **7** and their unnatural Δ^2-isomers, which lack a nitrogen-conjugated, α-positioned double bond and are almost

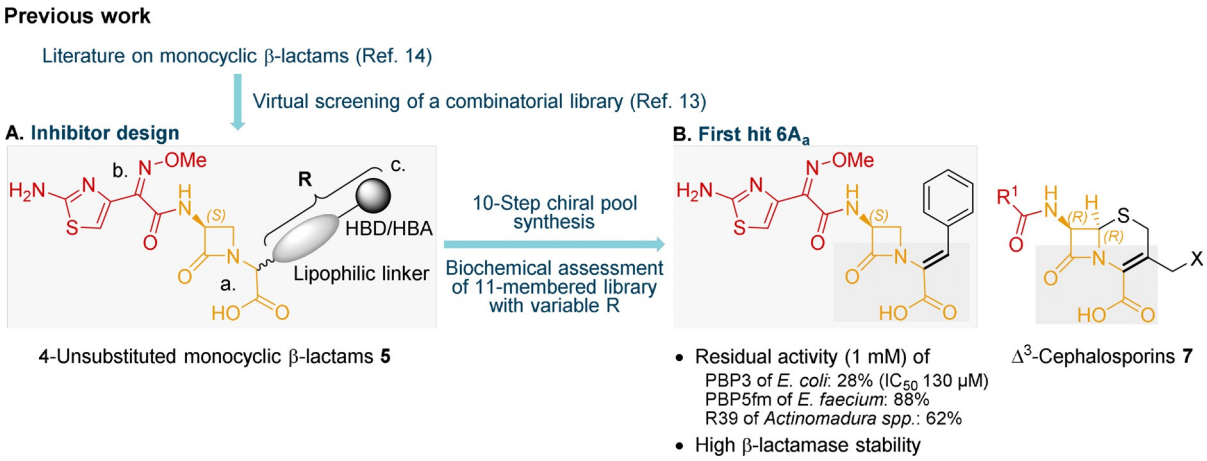


Figure 2. Previous work.[13,14] A. Design strategy. a. 3-Amino-1-carboxymethyl-β-lactam nucleus, mimicking the natural PBP substrate, D-alanyl-D-alanine, and enabling crucial molecular interactions with the enzyme's active site; b. Optimised 2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetamido substituent;[14] c. Variable side chain based on virtual screening, embedding a lipophilic linker and terminal hydrogen bond-donating or -accepting functionalities, enabling additional non-covalent interactions with the key residues in the enzyme's active site. B. Our first hit **6A$_a$**, its biochemical properties and structural resemblance to natural Δ^3-cephalosporins **7**.

completely devoid of activity (Figure 2B).[15] Encouraged by the fact that an appropriate lipophilic linker unit has been identified and considering compound **6A$_a$** as a key anchor point, a follow-up series is envisioned in the current work in which the chemical space around this interesting scaffold is further explored both from a synthetic and biological perspective.

Results and Discussion

Design

To initiate the hit expansion study and to further rationalise the above statement concerning the essentiality of α-unsaturation, in silico computations were invoked. Atomic Fukui indices were calculated for compound **6A$_a$** and compared to those of α-saturated analogue **8**, N1-unsubstituted compound **9** and nocardicin A **4A** (Figure 3A, for more information concerning this computational technique, see the Supporting Information). These condensed Fukui functions are local descriptors of site reactivity, predicting which atoms in the molecule are the most susceptible to electrophilic or nucleophilic attack, based on their intrinsic electronic properties.[16,17] As such, this technique can be used to compare the relative susceptibility of different sites within a molecule towards nucleophilic or electrophilic attack, or as a measure of how various side groups alter the reactivity. Generally, a nucleophilic reaction will preferably take place at the substrate site with the largest value for the LUMO density (indicated by the condensed Fukui function for nucleophilic attack at atom *k*, f_k^+), in this case the β-lactam carbonyl carbon atom. The hydroxyl warhead group of the active site serine residue has been assumed as the nucleophile or HOMO component of the nucleophilic addition reaction in this system. In that respect, recent studies have demonstrated that Fukui functions may indeed be a good metric *i.* to study β-lactam reactivity in the acylation step;[18] *ii.* to determine the reactivity of protein/ligand systems;[19] *iii.* to compare the reactivity of analogous sites across a series of related chemical species;[20] and *iv.* to study covalent binding of a wide range of inhibitors, including protease inhibitors.[21]

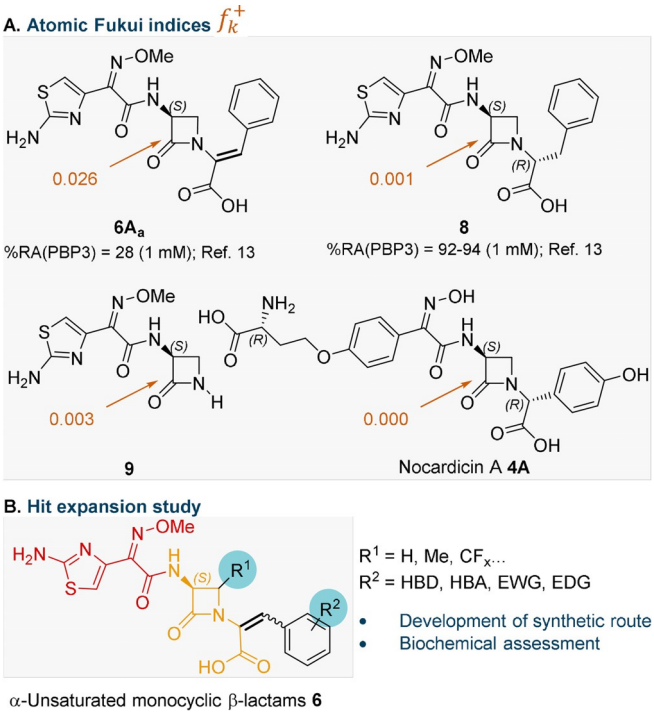


Figure 3. A. Atomic Fukui indices f_k^+ (absolute values) of the most electrophilic centre in related compounds **4A**, **6A$_a$**, **8**, **9**. B. Focus of this work: hit expansion of α-arylmethylidenecarboxylates **6**.

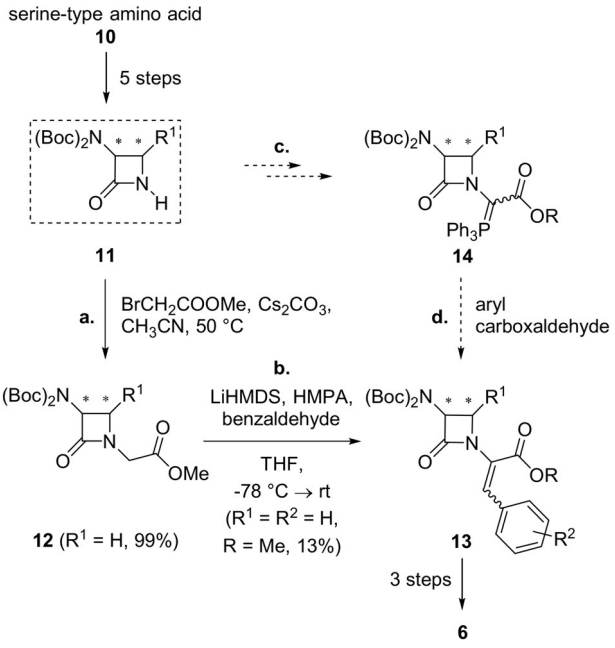
Clearly, the site-specific reactivity of benzylidene analogue **6A$_a$** ($f_k^+ = 0.026$) is higher than that of the related saturated analogue **8** ($f_k^+ = 0.001$), which is accurately correlated with the observed PBP3 residual activities (Figure 3A).[13] Furthermore, the values were found to be superior to those of N1-unsubstituted compound **9** and nocardicin A **4A**, suggesting that the α-unsaturated carboxymethyl moiety plays an activating role, additional to contributing to the D-Ala-D-Ala mimicry by the inhibitor. Experimental enzyme inhibition of course involves a complex interplay of multiple factors, including the overall geometry and interaction patterns of the inhibitors in the binding site. Still, the predicted chemical reactivity of the α-unsaturated 1-carboxymethyl-β-lactam scaffold and preliminary biological validation thereof were regarded here as a significant indication of the potential of this structural motif for further PBP inhibitor development. In that respect, it was considered to be of interest to assess the effect of the electron density of the α-side chain on the β-lactam chemical reactivity and affiliated biological impact through introduction of electron-withdrawing or -donating substituents 'R$^{2'}$ on the benzylidene aromatic ring (Figure 3B). Besides, additional non-covalent interactions with the PBPs' active site cavities are pursued in accordance with the original hydrogen-bonding requirements (Figure 2A).[13]

Apart from diversification of the benzylidene substitution pattern, it seemed interesting to evaluate the biological effect of different substituents 'R$^{1'}$ at the β-lactam C4-position while keeping the benzylidene ring intact. Introduction of a methyl moiety at that position is known to enhance the inhibitory activity against Gram-negative bacteria, while increasing the resistance against β-lactamase-associated decomposition of the β-lactam pharmacophore.[14] The FDA-approved monocyclic β-lactam pioneer aztreonam **3** well illustrates the latter aspect. On the other hand, it seemed also interesting to install a fluorine-containing motif at the β-lactam C4-position of target structures **6**. Approximately 25% of the pharmaceuticals on the market and within the development pipeline bears at least one fluorine atom, owing to its characteristic properties (small atom size, altering lipophilicity, high oxidative and thermal stability).[22,23] Most appealing for this work, however, are its electron-withdrawing capacities.[22] The latter property could confer an enhancement of the chemical reactivity of the neighbouring β-lactam carbonyl group, hence facilitating nucleophilic attack by the target enzyme serine residue, mostly realised in known monocyclic β-lactams by the N1-substituent.[14] As the target structures do not comprise a typical electron-withdrawing group at this position, the C4-substituent could be used for that purpose. Indeed, Bevilacqua et al. have demon-

strated that the half-life of a racemic *cis*-4-CF_3-monobactam in a biological buffer was lowered 85-fold compared to its 4-methyl analogue, demonstrating the increased chemical reactivity of the β-lactam nucleus.[24] As such, the incorporation of a tri-, di- or monofluoromethyl substituent seemed appropriate, as these groups have already been introduced in multiple performant medicines before (e.g., CF_3 in the blockbuster drug Prozac®).[22]

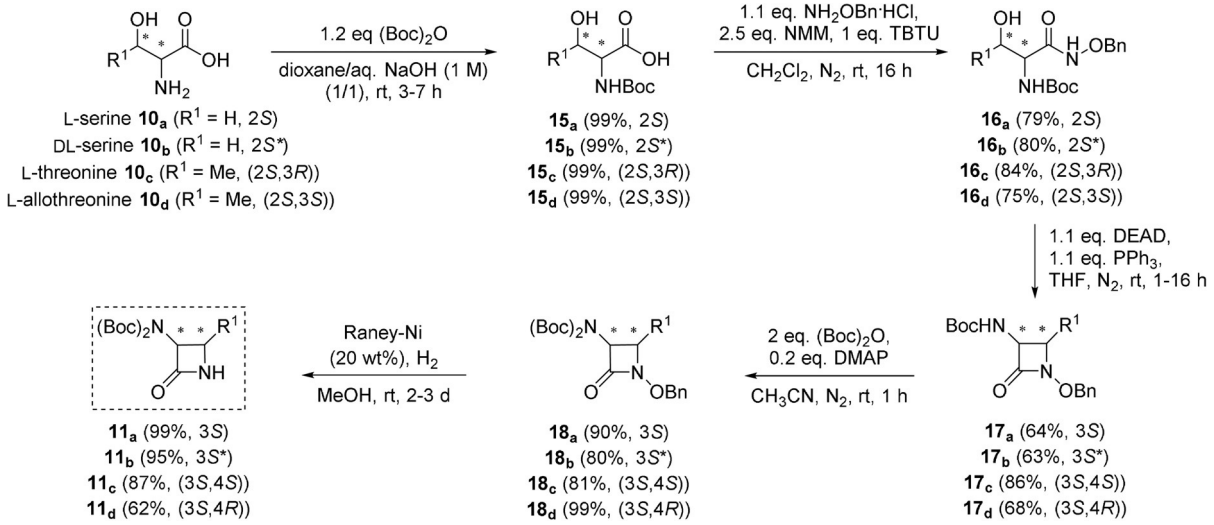
Synthesis

Taking into account the above-mentioned design features, an assortment of structurally diverse analogues of compound **6A$_a$** was prepared, elaborating on the previously developed, ten-step synthetic methodology starting from the amino acid serine.[13] However, the central steps of the latter pathway, starting from building block **11** and comprising lithium enolate functionalisation of the carbonyl α-position in ester **12** (steps **a–b**, Scheme 1), were adapted and optimised for the synthesis of α-unsaturated analogues in specific. One of the most robust and reliable tools for the formation of carbon-carbon double bonds concerns the Wittig olefination between aldehydes or ketones and Wittig reagents, ylides generated from phosphonium salts.[25] The intramolecular Wittig cyclisation has first been reported by R. Woodward in 1972, and has since been well established for the synthesis of carbapenem, cephem and penem β-lactam antibacterials.[26] On the contrary, the intermolecular counterpart for functionalisation of monocyclic β-lactams comprises a rather unexplored field, yet came forward as a suitable and versatile methodology, based on the wide variety of commercially available aldehydes and the advantage of late-stage introduction of the α,β-unsaturated functionality.[27] More specifically, the synthesis of α-arylmethylidene-substituted β-lactams **6** could be achieved through functionalisation of alkenes **13**. The latter could be prepared via Wittig olefination of suitable aryl carboxaldehydes with β-lactam ylides **14**, which in their turn originate from the same 3-amino-β-lactam building blocks **11** (steps **c–d**, Scheme 1).



Scheme 1. Previously developed lithium enolate functionalisation (**a**–**b**[13]) and alternative Wittig olefination strategy evaluated in this work (**c**–**d**).

In view of variation of the β-lactam C4-substituent, four new building blocks **11** were prepared using our established procedures (Scheme 2).[13] The biologically active form of the final compounds **6** is supposed to accommodate the 3′*S*-configuration. Both L- and DL-serine **10$_{a,b}$** were, however, employed for the synthesis of the corresponding 4-unsubstituted β-lactams **11$_{a,b}$** as to enable analysis of the optical purity of the target products **6** in a later stage. On the other hand, starting with L-threonine and L-allothreonine **10$_{c,d}$** allowed to efficiently obtain *trans*- and *cis*-4-methyl-β-lactam analogues **11$_{c,d}$**. More specifically, after quantitative Boc protection of these four easily available precursors **10**, hydroxamate synthesis was performed. A TBTU (*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate)-mediated coupling reaction using the



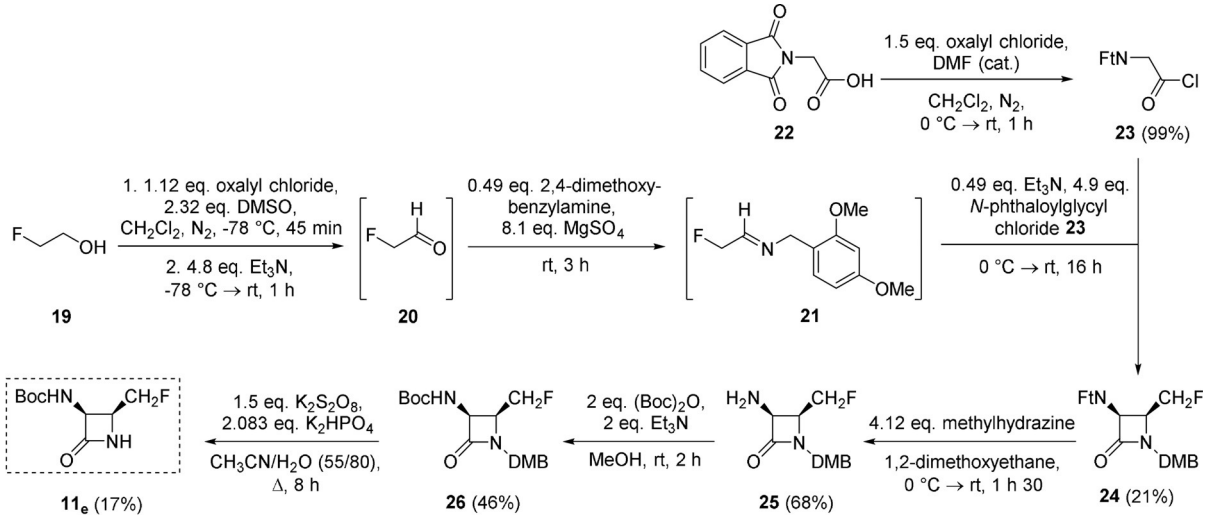
Scheme 2. Synthesis of di-Boc-protected 3-amino-(4-methyl)-β-lactams **11**.

hydrochloric acid salt of *O*-benzylhydroxylamine afforded propanamides **16**. Subsequently, cyclisation-prone substrates **16** were subjected to standard Mitsunobu conditions (diethyl azodicarboxylate, PPh_3), resulting in selective β-lactam ring formation. *N*-Benzyloxy-β-lactams **17** were provided with a second Boc group, after which Raney® nickel-catalysed hydrogenolysis released the amide nitrogen atom. As such, compounds **11** were produced in overall yields up to 50% and became available as building blocks for further functionalisation towards the target β-lactams **6**.

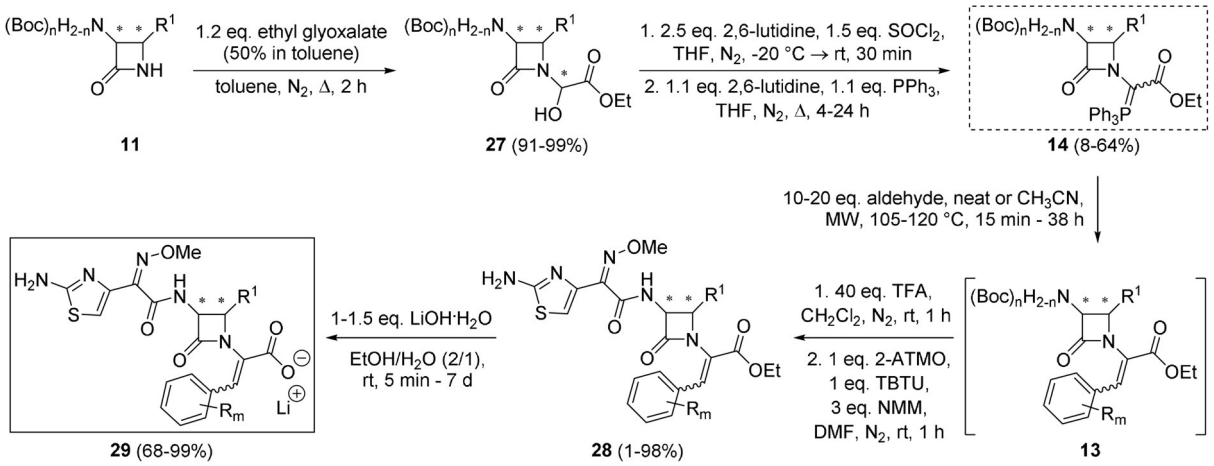
The preparation of trifluoro- and difluoromethyl-substituted β-lactams **6** from analogous building blocks **11** using the lithium enolate or Wittig reaction procedure was found to be highly complex due to excessive chemical reactivity. Decomposition or undesired side reactions, such as β-lactam ring opening, were often observed upon storing, handling or reacting these fluorinated compounds. Indeed, because of this very reason of the high reactivity induced by fluorine, our group has recently studied various transformation reactions of 4-trifluoromethyl-β-lactams.[28]

Still convinced by the introduction of fluorine as a means to increase the β-lactam electrophilicity, a monofluoromethyl building block $\mathbf{11_e}$ was targeted instead for use in the Wittig pathway. The relevance of a C4-fluoromethyl group in monocyclic β-lactams has equally been indicated in the literature before. According to Bevilacqua et al., 4-methyl- and 4-(fluoromethyl)monobactams show significant Gram-negative activity, opposed to trifluoromethyl analogues.[24] Moreover, the groups of Mitsuhashi and Ochiai have demonstrated that 4-(fluoromethyl)monobactams with varying aminothiazoleoxime side chains exhibit a comparable or even slightly superior inhibitory potency to aztreonam **3**, while significantly improving the stability against or inhibiting common β-lactamases, especially in a 3,4-*cis*-configuration.[29] For the synthesis of *cis*-4-fluoromethyl-β-lactam analogue $\mathbf{11_e}$, the Staudinger synthesis between a fluorinated imine **21** and a phthaloyl-protected aminoketene was applied, using conditions described by Yoshioka et al. (Scheme 3).[29] As fluoroacetaldehyde **20** is a non-commercial reactant, it was prepared from 2-fluoroethanol **19** via Swern oxidation, after which the intermediate aldehyde **20** was treated with 2,4-dimethoxybenzylamine in the presence of magnesium sulfate. Treatment of the crude imine **21** with *N*-phthaloylglycyl chloride **23**, synthesised from the corresponding amino acid **22**, in the presence of triethylamine furnished 4-fluoromethyl-β-lactam **24**. To avoid interference of the phthaloyl protecting group in later reaction steps, it was immediately removed from DMB-protected β-lactam **24** using methylhydrazine and replaced by the more common Boc group to form β-lactam **26**, which was subsequently submitted to oxidative cleavage of the DMB moiety using potassium persulfate to form the expected building block $\mathbf{11_e}$.

With this set of five building blocks **11** in hand, our alternative proposal consisting of Wittig olefination could be studied. To that end, after evaluation and optimisation of various procedures,[30,31] Boc-protected 3-aminoazetidin-2-ones **11** were condensed with ethyl glyoxalate in boiling toluene, quantitatively affording adducts **27** as equimolar mixtures of diastereomers (Scheme 4, Table 2). The need for purification was considered unnecessary by reducing the reported number of equivalents from three to 1.2, along with the addition of a minimal amount of toluene, sufficient to dissolve the starting material. The alcohols were subsequently chlorinated by means of thionyl chloride in alkaline environment, followed by S_N2-type substitution by triphenylphosphine in boiling THF. The presence of the base 2,6-lutidine caused the resulting phosphonium salts to be α-deprotonated and transformed into phosphoranes **14** in overall yields from **27** of up to 64%, hence giving rise to the central building blocks and setting stage for the subsequent Wittig olefination. Ylides **14** were obtained as mixtures of s-*cis*- and s-*trans*-isomers (d.r. = 53/47–86/14), the assignment of which was based on the effect of diamagnetic shielding of the ethoxy protons in the s-*trans*-configuration (^{1}H NMR, $CDCl_3$). Similar observations of the coexistence of two isomeric forms (at negative temperatures) of less functionalised ylides and a study on their reactivity have been reported by Kayser and Hatt.[32]



Scheme 3. Synthesis of Boc-protected 3-amino-4-fluoromethyl-β-lactam **11e**.



Scheme 4. Synthesis of α-arylmethylidene-substituted (4-(fluoro)methyl)-β-lactams **29**.

Based on reported procedures for the (intramolecular) Wittig reaction, a number of exploring experiments was performed in order to condense ylide **14$_a$** with (distilled) benzaldehyde as a model aldehyde, aspiring α-benzylidene-substituted, di-Boc-protected 3-amino-1-ethoxy-carbonylmethyl-β-lactams **13A$_a$** (R_m = H, Scheme 4, Table 1, entries 1–4).[26,27] Firstly, β-lactam ylide **14$_a$** and benzaldehyde were merged in the presence of a catalytic amount of hydroquinone and stirred at high temperature in xylenes. Gradually, additional equivalents of benzaldehyde, reaching a large excess in the end, were added to stimulate the slow conversion towards alkene **13A$_a$**. Due to the long residence time at high temperature, multiple side products were formed along with the target product (entry 1). Changing the solvent to acetonitrile enabled a more rapid reaction, though still required a large excess of the aldehyde (entry 2). Lowering the amount of aldehyde, while allowing a longer reaction time in toluene, caused a complex mixture to form with incomplete transformation of the starting material (entry 3). In order to avoid the potential equilibration of compound **14$_a$** with its conjugate acid due to the presence of proton traces in the reaction medium, possibly quenching the reactive ylide and discouraging its conversion, a stoichiometric amount of 2,6-lutidine was added as a proton sponge.[32] After two days of stirring under reflux conditions in toluene, the conversion was still only moderate (entry 4). Similar results were obtained using other functionalised aldehydes and ketones. Apparently, the Wittig reaction required harsh conditions to reach complete conversion of the sterically demanding starting material and definitely needed a boost to improve the reaction rates. Sonochemistry, for example, uses ultrasound waves to cause cavitation, a phenomenon known to liberate considerable amounts of energy, thus causing spectacular rate-enhancing effects in homo- and heterogeneous reaction mixtures.[33] Hence, the use of sonication waves, in combination with up-concentration of the medium to accelerate the conversion of the starting material, was attempted, but was equally in vain (entry 5). A logical follow-up experiment concerned heating of the reaction mixture using a microwave reactor (entries 6–8).[34] Microwave technology allows to perform chemical reactions under controlled conditions in combination with efficient heating through absorption of microwave energy.[35] The technique is acknowledged for enabling high-speed synthesis, dramatically reducing reaction times from days and hours to minutes and even seconds.[36] Indeed, upon microwave-assisted heating of a concentrated mixture of β-lactam **14$_a$** and benzaldehyde in acetonitrile, a moderate, and notably selective conversion was reached after only four hours (entry 6). Random streamlining of the reaction parameters (entries 7 and 8) permitted to

Table 1. Exploration of the Wittig olefination between β-lactam ylide **14$_a$** (0.08 mmol) and benzaldehyde.[a]

Entry	Aldehyde	Additive	Solvent (conc.)[b]	T (°C)	Time	Conversion to **13A$_a$**[c]
1	2.5–30 equiv.	cat. hydroquinone	xylenes (0.05 M)	84 °C	96 h	100%, complex mixture
2	2.5–30 equiv.	–	CH_3CN (0.05 M)	Δ	24 h	100%, complex mixture
3	1.05–4 equiv.	–	toluene (0.05 M)	Δ	4 d	71%, complex mixture
4	1 equiv.	1 equiv. 2,6-lutidine	toluene (0.15 M)	Δ	48 h	50%, complex mixture
5	4 equiv.	–	CH_3CN (0.15 M)	)))[d], 70 °C	3 h	0.5%
6	4 equiv.	–	CH_3CN (0.25 M)	MW, 120 °C[e]	4 h	42%, selective[f]
7	1 equiv.	–	toluene (0.15 M)	MW, 120 °C[e]	36 h	81%, selective[f]
8	10 equiv.	–	neat	MW, 120 °C[e]	50 min	100%, selective[f]

[a] Distilled benzaldehyde is used. [b] Concentration of ylide **14$_a$** in the corresponding solvent. [c] Conversion of starting material **14$_a$** to alkene **13A$_a$**, determined via LC-MS analysis. [d] Application of ultrasound waves. [e] Operating power: 150 W. [f] Selective conversion of starting material to expected product **13A$_a$**.

eventually obtain complete conversion of the starting material after 50 minutes using an excess of benzaldehyde under neat conditions.

The microwave-assisted synthesis of alkenes **13** was therefore studied in-depth. Various reaction parameters were screened to attain the most efficient olefination of ylides **14**, hence enabling reaction scale-up and expansion of the reactant scope. Various solvents were examined since neat conditions resulted in complete conversion of the starting material in the presence of liquid benzaldehyde, but caused insufficient mixing of solid aldehydes or ketones, inducing thermal decomposition of the reaction medium. Appropriate solvents were selected, governed by their dielectric properties and hence their ability to absorb microwave energy and convert it into heat.[36] Out of a range of solvents with divergent microwave-absorbing capacities, ethylene glycol was found to perform the best, followed by DMF, ethanol and acetonitrile. Next, the optimal reaction temperatures were set for ethylene glycol (120 °C) and acetonitrile (105 °C), based on a trade-off between higher conversion rates and commencing formation of side products with a raise in temperature. Aiming at a maximal enhancement of the conversion rate, the number of equivalents of benzaldehyde was also studied. The cleanest reaction mixture was obtained when the reaction time was kept as short as possible. As such, the use of a large excess of benzaldehyde appeared to be the most convenient, as the reaction media were planned to be further used without intermediate purification and to be directly submitted to acidic environment for deprotection of the 3-amino group (Scheme 4). As the resulting amine-TFA salts were anticipated to be water-soluble, a simple extraction procedure could be used to remove the excess of aldehyde, remaining starting material and by-product triphenylphosphine oxide via the organic phase. It must be noted, though, that in several literature reports, the ylide component is added in excess.[26,27] This was not practically feasible in this case, as ylide $\mathbf{14_a}$ was obtained only after eight reaction steps, putting a severe restriction on the amount of reactant available, while the use of an excess of ylide $\mathbf{14_a}$ would not be consistent with the purification strategy. With optimal reaction parameters in hand, several other aldehydes and ketones were screened to extend the reactant scope, envisioning a series of novel α-arylmethylidene-substituted β-lactam esters **13** (Table 2). When selecting the appropriate carbonyl compounds, our design requirements were kept in mind (lipophilic linker, terminal HBD/HBA or EWG/EDG, Figure 3B).

As such, based on their structural properties, satisfactory conversion rate and the purity of the obtained reaction media, five additional derivatives were selected for further functionalisation towards the end products (aryl substituent R_m: 2,6-diF, 4-CN, 4-NO_2, 4-CF_3, 4-OMe). Further fine-tuning of the reaction parameters was performed for each compound and the other building blocks $\mathbf{11_{b-e}}$ before scaling up the reactions. For example, ethylene glycol was replaced by acetonitrile in some cases based on the solubility of the reactants or the high viscosity of ethylene glycol, hampering acceptable reactant mixing. Remarkably, Wittig olefination using the electron-rich *p*-anisaldehyde or C4-substituted β-lactam ylides required considerably higher reaction times to obtain full conversion. The final, optimised conditions for condensation of ylides **14** with the functionalised aldehydes are displayed in Scheme 4 and Table 2. As such, the microwave-assisted Wittig olefination reactions efficiently afforded the crude olefins **13** after evaporation of the reaction media in vacuo, after which TFA-mediated Boc removal was performed (Scheme 4, Table 2). As anticipated, the amine-TFA salts were obtained in a sufficiently pure form after removal of the majority of the organic solvent-soluble by-products, excessive aldehyde and impurities through liquid–liquid extraction. Coupling of the released amines with the 2-ATMO scaffold applying our established procedures,[13] efficiently furnished functionalised ethyl esters **28**. Straightforward LiOH-assisted hydrolysis of ethyl esters **28** in an ethanol/water (2/1) mixture eventually afforded lithium carboxylates **29** in quantitative yields. The lithium carboxylates **29** were later neutralised (1 M HCl) to afford carboxylic acids **6** for evaluation of their inhibitory potential on various PBPs. A final overview of the target structures **6** hence obtained, is provided in Figure 4. This series was completed by addition of 4-pyridinylmethylidene analogue $\mathbf{6G_a}$, which was previously synthesised in ten steps by means of the lithium enolate functionalisation pathway (reaction procedures identical to previously reported work, ±1% overall yield, Scheme 1).[13]

From a mechanistic point of view, an arsenal of possibilities has been proposed during the last half-century to explain the condensation of ylides and carbonyl compounds. In this context, a recent overview has been provided by Byrne and Gilheany.[37] The mechanism of the Wittig reaction remains a controversial topic, in which the origin of stereoselectivity is still not fully understood and multiple exceptions arise. Broadly speaking, the nature of the ylide dictates the stereochemical outcome of the reaction based on the degree of anion stabilisation by the ylide α-substituent [*i.* non-stabilised ylides ($R_3P=CHR'$ with R′ = alkyl) lead to *Z*-alkenes; *ii.* semi-stabilised ylides (R′ = conjugated C–C double bond) convey low selectivity; *iii.* stabilised ylides (R′ = EWG) generate *E*-alkenes]. Prediction of the stereochemistry is, however, not always straightforward and may be complicated by several factors. Ylides **14** are stabilised by the presence of the electron-withdrawing ester moiety, postulated to afford alkenes with the stabilising alkoxycarbonyl moiety *trans* to the aldehyde aryl group.[37,38] Enriched mixtures of two isomers were obtained in most cases.[1] In this respect, 2D NOESY NMR experiments were invoked to assign the *E*/*Z*-stereochemistry to the isomeric structures **28**, as the configuration of the double bond could not be derived from routine NMR experiments and, based on the non-crystalline state of the compound, X-ray analysis could not give a decisive answer regarding the stereochemistry. Using the same technique as previously described,[13] NOESY experiments (CD_3OD) were performed to define interactions between crucial H-atoms in space. Both geometrical isomers of each com-

[1] *The isomeric ratio could only be determined at the level of 3-acylamino-β-lactams* ***28****, that is, after two additional reaction steps and single- or two-step chromatographic purification. A slight deviation from the isomeric ratio inherent to the Wittig reaction might therefore be possible.*

Table 2. Synthesis of ylides **14**, their Wittig reaction (0.5 mmol) with aryl carboxaldehydes ($(C_6H_{(5-m)}R_m)CHO$) and functionalisation towards carboxylates **29A–F**.

Cpd[a]	R^1	*n*	**27** (%)	Stereo	d.r.	**14** (%)	d.r.[d]	Wittig olefination: aldehyde (equiv)	R_m	solvent (conc.)[e]	*T* (°C)[n]	Time	**28** (%)	d.r. (*E*/*Z*)[j,k]	*ee*[m]	**29** (%)	d.r. (*E*/*Z*)[k]
$\mathbf{11_a}$	H	2	97	3′*S*	48/52	60[b]	37/63	10	H	ethylene glycol	120	30 min	$\mathbf{28A_a}$ (26)[b]	4/96[b]	>90	$\mathbf{29A_a}$ (99)	0/100
$\mathbf{11_b}$	H	2	99	3′*S**	46/54	30[b]	40/60			(0.8 M)			$\mathbf{28A_b}$ (8)[b]	13/87[b]	0	$\mathbf{29A_b}$ (99)	7/93
$\mathbf{11_a}$	H	2	97	3′*S*	48/52	60[b]	37/63		4-CN	CH_3CN (0.4 M)	105	40 min	$\mathbf{28B_a}$ (41; 17)[f]	47/53; 64/36[f]	–	$\mathbf{29B_a}$ (99)	69/31
$\mathbf{11_a}$									4-NO_2	CH_3CN (0.2 M)		60 min	$\mathbf{28C_a}$ (98; 16)[f]	22/78; 29/71[f]	–	$\mathbf{29C_a}$ (99)	20/80
$\mathbf{11_a}$									2,6-diF	CH_3CN (0.8 M)		30 min	$\mathbf{28D_a}$ (61; 5)[f]	14/84; 14/84[f,l]	–	$\mathbf{29D_a}$ (99)	0/100[l]
$\mathbf{11_a}$									4-CF_3	ethylene glycol	120	15 min	$\mathbf{28E_a}$ (20; 6)[f]	47/53; 47/53[f]	>87	$\mathbf{29E_a}$ (99)	23/77
$\mathbf{11_b}$	H	2	99	3′*S**	46/54	30[b]	40/60			(0.4 M)			$\mathbf{28E_b}$ (50)[b]	22/78[b]	0	$\mathbf{29E_b}$ (99)	0/100
$\mathbf{11_a}$	H	2	97	3′*S*	48/52	60[b]	37/63		4-OMe	CH_3CN (0.4 M)	105	13 h	$\mathbf{28F_a}$ (12)[g]	6/94[g]	–	$\mathbf{29F_a}$ (99)	0/100
$\mathbf{11_c}$	Me	2	94	3′*S*,4′S	43/57	28[b]	14/86		H	neat	120	30 h	$\mathbf{28A_c}$ (4)[b]	42/58[b]	100	$\mathbf{29A_c}$ (99)	17/83
$\mathbf{11_d}$	Me	2	91	3′*S*,4′*R*	47/53	8[c]	30/70					6 h	$\mathbf{28A_d}$ (8)[c]	35/65[c]	100	$\mathbf{29A_d}$ (79)	29/71
$\mathbf{11_c}$	Me	2	94	3′*S*,4′*S*	43/57	28[b]	14/86	15	4-NO_2	CH_3CN (0.16 M)	105	38 h	$\mathbf{28C_c}$ (<1)[h]	–	100	–	–
$\mathbf{11_d}$	Me	2	91	3′*S*,4′*R*	47/53	8[c]	30/70	20				14 h	$\mathbf{28C_d}$ (18)[h]	32/68[h]	100	$\mathbf{29C_d}$ (68)	26/74
$\mathbf{11_e}$	CH_2F	1	99	3′*S**,4′*S**	48/52	64[b]	47/53	15	H	CH_3CN (0.8 M)		10 h	$\mathbf{28A_e}$ (1)[i]	36/64[i]	0	$\mathbf{29A_e}$ (70)	32/68

[a] The subscript "a–e" refers to the stereochemistry and C4-substitution, see Scheme 2. [b] After automated column chromatography (C18). [c] After column chromatography (SiO_2) and automated column chromatography (C18). [d] Ratio of s-*trans*- and s-*cis*-isomers. [e] Concentration of ylide **14** in the corresponding solvent. [f] After (automated) column chromatography (C18/SiO_2); after additional prep. TLC. [g] After automated column chromatography (SiO_2). [h] After preparative HPLC. [i] After automated column chromatography (C18), prep. TLC and prep. HPLC. [j] Isomeric ratio after purification. Isomeric ratio before purification could not be determined due to complexity of ^{1}H NMR and LC-MS spectra. [k] Determined via ^{1}H NMR analysis (MeOD-d_4 or D_2O). Major isomer has the 2*Z*-stereochemistry, as determined via NOESY-analysis. [l] *E*/*Z*-stereochemistry not to be determined due to the absence of significant NOE interactions (MeOD-d_4 or D_2O). [m] For R^1 = H: determined via chiral HPLC. Separation conditions; ChiralPak® IA column; 0.5 mL min^{-1}; 35 °C; hexane/EtOH (88/12); 300.8 nm (for **28A**); ChiralPak® IA column; 0.3 mL min^{-1}; 35 °C; hexane/*i*PrOH (50/50); 300.8 nm (for **28E**); For R^1 = Me: determined via ^{1}H NMR analysis (MeOD-d_4). [n] MW at 150 W.

pound **28** were presented in 3D-format, after which energy-minimisation permitted to make an educated guess regarding the configuration, based on comparison with the experimental NOE-effects. An example of the methodology is given in the Supporting information section for compound $\mathbf{28A_a}$. Except for 2,6-difluoro-substituted derivative **28D**, in which no significant NOE interactions could be observed between the concerned protons (aromatic, C4- and alkene protons) due to the presence of fluorine atoms on crucial positions, the predominant isomer was indeed assigned the 2*Z*-stereochemistry in all other cases (aryl *trans* to ester moiety). Despite the fact that the synthesis of tri- and tetrasubstituted alkenes via reactions of α(α-di)-substituted phosphoranes with aldehydes or ketones is described to confer poor yields and low *E*/*Z*-selectivities,[39] trisubstituted alkenes **13** were obtained here in high conversion rates and with moderate to high stereoselectivities.

Finally, assessment of the optical purity of esters $\mathbf{28A_a}$ and $\mathbf{28E_a}$ via chiral HPLC demonstrated conservation of the configuration of the 3′*S*-chiral centre throughout the entire pathway, translated in high enantiomeric excess values (*ee* >87–90%, Table 2). Hereto, the hydrolysis step, demonstrated previously to ensue enantiospecifically by means of a chiral resolution strategy,[13] was taken into account. For 4-methyl-β-lactams $\mathbf{29_{c,d}}$, the occurrence of C3-epimerisation would easily be derivable from the *cis*/*trans*-ratio, to be determined on the basis of the value of the coupling constant of the doublet signal belonging to the C3-proton (^{1}H NMR, CD_3OD, $\mathbf{29_{c,d}}$: *J* = 2–3 Hz (*trans*); *J* = 4–5 Hz (*cis*)).[40] Satisfactorily, the expected *cis*- or *trans*-epimers (the 3′*S*-isomers) were formed selectively.

Although the enantiopurity of compound $\mathbf{6A_a}$ obtained via lithium enolate functionalisation as the crucial reaction step (ten steps, 1–4%) was even higher (*ee* >96%),[13] the current overall yield of $\mathbf{6A_a}$ was found to be significantly elevated. Indeed, compounds **6A**–**F** were efficiently obtained in 12–13 reaction steps in global yields up to 26%, presenting the latter protocol as the most profitable choice for the synthesis of α-arylmethylidene-substituted β-lactams **6**. Apart from the specific isomeric ratios, the spectral data of *Z*-arylmethylidene derivatives $\mathbf{29A_a}$ prepared via the two different methodologies, were judged to be identical.

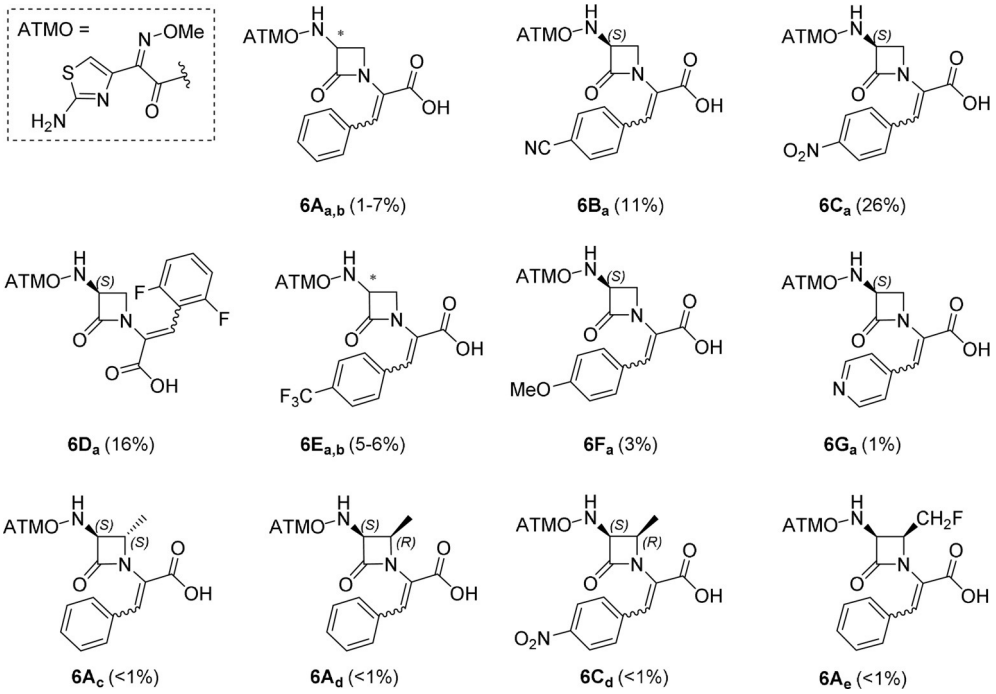
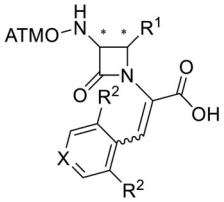


Figure 4. Overview of the newly synthesised second-generation structures **6** with varying aromatic and C4-substituents.

Biochemical assessment

The potential of the newly prepared libraries of functionalised (C4-substituted) α-arylmethylidenecarboxylates **6** to inhibit various (resistant) PBPs was assessed and compared to that of the parent structure **6A$_a$** (Table 3). A PBP binding and competition assay was performed using the purified PBP3 of *E. coli* K12, PBP5fm of *Enterococcus faecium* D63r and R39 DD-carboxypeptidase of *Actinomadura* spp., after which the residual enzymatic activity of the enzymes was determined.[41] The first series

Table 3. Residual enzymatic activities after incubation of PBP3, PBP5fm and R39 (2.5 μM) with selected compounds **6** (1 mM, pH 7, 30 °C, 3 h), IC_{50} values on PBP3/R39 and predicted f_k^+ of selected compounds.



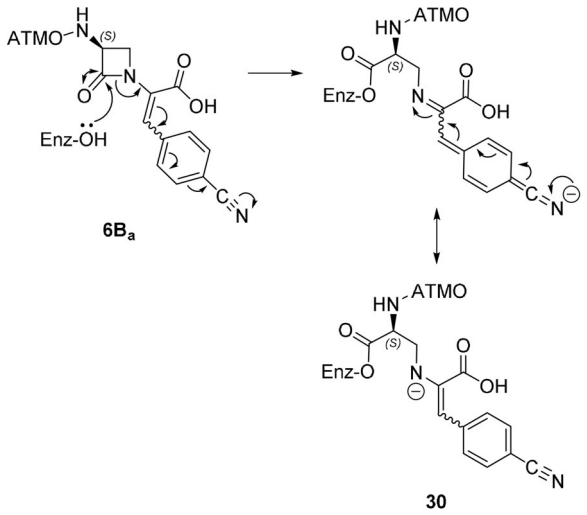
Cpd	R^1	R^2	X	Stereo (*E*/*Z*)[a]	%RA PBP3 (IC_{50}, μM)	%RA PBP5fm	%RA R39 (IC_{50}, μM)	f_k^+[e]
Azn 8A	–	–	–	–	– (0.004–0.022)[b]	–[c]	– (4)[d]	–
blank	–	–	–	–	100	100	100	–
6A$_a$	H	H	C(H)	3′*S* (0/100)	28 (130)	88	62	0.026
6B$_a$	H	H	C(C≡N)	3′*S* (69/31)	40	100	6 (58)	0.031
6C$_a$	H	H	C(NO_2)	3′*S* (20/80)	47	100	2 (20)	–
6D$_a$	H	F	C(H)	3′*S* (0/100)	11	100	53	0.039
6E$_a$	H	H	C(CF_3)	3′*S* (23/77)	2	100	7 (122)	0.045
6F$_a$	H	H	C(OMe)	3′*S* (0/100)	20	100	23	0.026
6G$_a$	H	H	N	3′*S* (0/100)	86	100	39	0.035
6A$_c$	Me	H	C(H)	3′*S*,4′*S* (0/100)	26	100	89	–
6A$_d$	Me	H	C(H)	3′*S*,4′*R* (29/71)	69	100	91	–
6C$_d$	Me	H	C(NO_2)	3′*S*,4′*R* (19/81)	16 (47)	100	100	–
6A$_e$	CH_2F	H	C(H)	3′*S**,4′*S** (32/68)	75	100	92	0.029

[a] *E*/*Z*-configuration of arylmethylidene double bond, as suggested via NOESY experiments. Only for **6D$_a$**, no proposal could be made. [b] Computed from literature data.[42] [c] Aztreonam **3** is not active against Gram-positive bacteria. [d] Computed from literature data.[43] [e] Nucleophilic condensed Fukui function for selected compounds (2*Z*-configuration), absolute values.

comprised direct analogues of compound **6A$_a$**, in which the substitution pattern of the benzylidene aromatic ring was varied through inclusion of electron-donating or -withdrawing groups, mostly endowed with hydrogen-bonding capacities (Figure 4). None of the seven compounds **6** synthesised was able to significantly inhibit the highly resistant PBP5fm. On the contrary, the potential of the compounds to inhibit both other enzymes (PBP3 and R39) indeed seemed to benefit from additional substitution of the aromatic ring as the residual activity percentages (especially those of R39) dropped in comparison to those of compound **6A$_a$** (e.g., 4-OMe-, 4-CF_3-, 2,6-diF-substitution for PBP3, supplemented with 4-NO_2- and 4-CN-substitution and the switch to the pyridine-based system for R39). Especially 4-nitro analogue **6C$_a$** displays an interesting activity profile (%RA(R39) = 2%; IC_{50} = 20 μM) and might constitute a valuable scaffold for advanced follow-up research on PBP inhibitors.

The increase in potency compared to parent compound **6A$_a$** (especially for R39) could be partly attributed to enhanced electrophilicity of the scaffold based on the increased electron-withdrawing ability of the side chain. To probe the electrophilicity of the β-lactam carbonyl carbon atoms in this focused compound library, atomic Fukui indices were again calculated for selected compounds (Table 3). Expectedly, the site-specific electrophilicity values (expressed by the condensed nucleophilic Fukui function) found for the derivatives substituted with electron-withdrawing groups **6B**,**D**,**E**,**G$_a$** (f_k^+ = 0.031–0.045) are higher than those of the parent compound **6A$_a$** (f_k^+ = 0.026). Indeed, for R39, an increase in f_k^+ generally implies a decrease in the residual activity of the enzyme, although the relationship is not linear. The lack of a true correlation emphasises the involvement of other important factors in enzyme inhibition besides mere chemical reactivity, such as the overall geometry and interaction patterns of the inhibitors in the binding site by means of, for example, hydrogen bonding (e.g., X = N, C(C≡N), C(NO_2), C(OMe)…). The reason for enhanced activity and the importance of the specific substituents with regard to additional non-covalent interactions and the positioning of the pharmacophore within the respective catalytic sites might be derived from experimental X-ray data of co-crystallised complexes with the inhibitors. In that respect, co-crystallisation studies of PBP3 and R39 with compounds **6B-G$_a$** are ongoing. Lastly, the very low R39 residual activity values for **6B**–**C$_a$** might be attributed to expansion of the PCMO. The nitro and cyano groups could for example act as electron sinks upon opening of the β-lactam ring by the nucleophilic serine residue, as proposed in Scheme 5.

The next set of compounds is composed of 4-substituted α-arylmethylidene-substituted 1-carboxymethyl-β-lactams **6$_{c–e}$** and mainly involves variation of the β-lactam C4-position (Figure 4, Table 3). As a 4-methyl group has been reported to increase the stability of monocyclic β-lactams (*cfr.* aztreonam **3**, a *trans*-configured monobactam) against common β-lactamases and to enhance the Gram-negative activity,[14] the biological effect of the introduction of a methyl group in both the *cis*- and *trans*-configuration was investigated. *trans*-4-Methyl analogue **6A$_c$** showed comparable PBP3 inhibition (26% RA) as



Scheme 5. Increased reactivity of the inhibitor attributed to expansion of the PCMO and resonance stabilisation (for **6B$_a$** as an example).

the original compound **6A$_a$** (28% RA, IC_{50} = 130 μM) and a higher potency than the *cis*-analogue **6A$_d$**, though very poor PBP5fm and R39 activity. Notably, introduction of an aromatic nitro group in *cis*-analogue **6A$_d$**, giving rise to compound **6C$_d$**, significantly enhanced the inhibitory potential against PBP3 (%RA from 69 to 16%, IC_{50} = 47 μM). Probably, the effect would be comparable for the corresponding *trans*-analogue **6C$_c$**, for which, unfortunately, the isolated amount of precursor **28C$_c$** was insufficient for further transformation and biochemical evaluation. The residual activity values determined after preincubation with the racemic *cis*-4-fluorinated derivative **6A$_e$** were not noteworthy and rather paralleled those of *cis*-4-methyl analogue **6A$_d$**. It must be noted, though, that the effects provoked by the latter would be more pronounced if the derivative could be isolated or produced in an enantiomerically pure form, for example by using (*R*)-phenylethylamine as a chiral template instead of 2,4-dimethoxybenzylamine in the Staudinger synthesis.[44]

Secondly, the stability of β-lactams **6A$_{a,c,d}$** against β-lactamase-catalysed hydrolysis was determined using a colourimetric assay (Table 4). The residual activity of representative enzymes of each of the four β-lactamase classes was measured

Table 4. Residual enzymatic activities after incubation of different β-lactamases with compounds **6A$_{a,c,d}$** (1 mM, 37 °C, 1.5 min).

Class	Enzyme	%RA after incubation with	
		6A$_a$	**6A$_c$**
A	CTX-M15	103	118
B	Imp1	109	94
B	Imp4	97	95
C	P99	61	107 (84[a])
C	AmpC-HD	109	108
D	Oxa48[b]	98	70
D	Oxa163	–	82

[a] After incubation with **6A$_d$**. [b] Oxa48 is not inhibited by MeOH (1%): 99 ± 10%.

after incubation in the presence of the compounds by following the rate of hydrolysis of nitrocefin as a chromogenic reporter substrate.[45] While the introduction of a *cis*-4-methyl group into parent compound **6A$_a$** slightly compensated for the loss of catalytic activity of class C enzyme P99 (84% RA for **6A$_d$** as opposed to 61% for the native compound **6A$_a$**), a shift was noticed for *trans*-4-methyl-β-lactam **6A$_c$** from inhibition of class C to class D β-lactamases. The latter is therefore not entirely in agreement with the postulation that C4-methylation systematically entails a higher β-lactamase stability. Compound **6A$_c$** weakly inhibited class D Oxa48 (70% RA). Oxa48 and its variants are widespread in *K. pneumoniae* and other *Enterobacteriaceae* and *A. baumannii* and demote the clinical efficacy of carbapenems.[46] To confirm the observed effect, the potential inhibition by methanol (1%), used for solubilisation of the compound, was excluded. Meanwhile, weak inhibition of a second class D representative, Oxa163, a less efficient carbapenem- but effective aztreonam-hydrolysing enzyme, was demonstrated (82% RA).[46] The specific mode of inhibition remains unclear for the time being. Most importantly, it can be concluded that the compound was not significantly affected by any of the β-lactamases tested, which parallels the results of the 4-unsubstituted counterpart **6A$_a$** and those of the nocardicins **4** in general.[47]

Overall, it can be stated that the introduction of a *trans*-4-methyl moiety seemed especially useful for PBP3 inhibitors, while functionalisation of the aromatic ring with electron-withdrawing or hydrogen-bonding groups rendered the α-benzylidene-substituted 3-acetamido-1-carboxymethyl-β-lactam scaffold more capable of inhibiting both PBP3 of *E. coli* and R39 of *Actinomadura* spp. Specific α-benzylidene-substituted inhibitors have additionally proven their stability against β-lactamase-promoted β-lactam ring cleavage, while weakly inhibiting class C or class D representatives. As such, the significance of α-unsaturation and electron-withdrawal of the connected side chain for provoking a PBP or β-lactamase inhibitory effect has been validated and represents an important insight, definitely to be taken into account for further monocyclic β-lactam inhibitor design.

Conclusions

β-Lactam antibiotics have revolutionised healthcare by enabling effective treatment of numerous infectious diseases and forming the bedrock of many of the greatest medical advances of the past century. The azetidin-2-one-containing drugs, targeting the bacterial penicillin-binding proteins, had their heyday in the 1950s. Today, however, the dissemination of resistance mechanisms throughout pathogenic microbes combined with the inability to launch new antibiotics on the market at the same pace, is gradually eroding their usefulness. If left unchecked, this trend could soon grow into a crisis of global scale. Keeping in mind the long-time demonstrated potency, safety and efficacy of β-lactams, but moving away from extensively scrutinised bicyclic β-lactams, we studied their monocyclic counterparts. The latter are less renowned, though effectively acylate PBPs in the same way and intrinsically resist degradation by or even inhibit β-lactamases. Based on the potential of our previously developed hit compound **6A$_a$** to inhibit *E. coli* PBP3, while resisting β-lactamase-mediated degradation, α-benzylidene-substituted 3-amino-1-carboxymethyl-β-lactams were considered to be valuable structures in the battle against (resistant) bacteria. Endorsed by calculated Fukui indices, additional SAR studies around benzylidene-like compounds seemed appropriate to assess the effect of small variations around the basic hit scaffold in the search for more potent inhibitors. Both aiming at increased β-lactam electrophilicity, an induced electron flow after enzymatic β-lactam ring opening and additional non-covalent interactions with the PBPs' active site cavities, the substitution pattern of the benzylidene aromatic ring was explored in the first instance via incorporation of electron-donating, electron-withdrawing or hydrogen-bonding groups. Additionally, the hit evolution study focused on the preparation of C4-methyl- and C4-fluoromethyl-β-lactams, envisioning further enhancement of the PBP inhibitory potency and β-lactamase stability.

After straightforward preparation of a set of (C4-substituted) 3-amino-β-lactam building blocks from easily available starting materials, a new synthetic functionalisation route was developed. The Wittig olefination between aldehydes and phosphonium ylides, one of the most robust and reliable tools for C–C double bond formation, emerged as the most crucial step of the pathway and proved to be a viable option for the preparation of α-unsaturated 1-carboxymethyl-β-lactams. To that end, β-lactam ylides were synthesised in a few steps starting from building blocks **11**, followed by their microwave-assisted Wittig olefination and further functionalisation. The target compounds could be obtained in twelve steps in up to 26% overall yield (a 13-fold improvement compared to our previous work) and high enantiomeric excess (*ee* $>$ 87%).

Preliminary assessment of the PBP3, PBP5fm and R39 inhibitory potency of this second-generation library confirmed the potential of α-unsaturated carboxylates as a novel class of monocyclic β-lactams. The beneficial impact on PBP3 inhibitory potency of incorporation of a *trans*-4-methyl group into hit structure **6A$_a$** was proven, while the β-lactamase stability (with slight inhibition of class D representatives) was shown to be maintained. The most significant PBP3/R39 potency enhancement was, however, achieved through functionalisation of the α-arylmethylidene aromatic ring. For example, nitro derivative **6C$_a$** provoked a residual activity of only 2% after incubation with R39, culminating in an IC_{50} of 20 μM. The introduction of certain electron-withdrawing groups in specific indeed seemed valuable based on the potential increase in β-lactam electrophilicity and the suggested effect of resonance stabilisation of the amide nitrogen anion formed after nucleophilic attack by the target serine residue. Besides the ongoing co-crystallisation experiments with PBP3 or R39, additional investigations into the SAR landscape are recommended to gain insight in the specific role of these structural features. The side chain stereochemistry, linker unit length and substitution pattern could be further examined in order to leverage favourable binding site interactions and increase inhibitory potency. As such, the chemical pathway elucidated herein might be

used to further guide valorisation and hit-to-lead development.

In summary, an elaborate chiral pool synthetic protocol was established for the preparation of a variety of novel α-arylmethylidene-substituted 3-amino-1-carboxymethyl-β-lactams. Overall, these α-unsaturated carboxylates were proven to be eligible lead structures towards a new class of monocyclic β-lactam antibacterials, which remain highly underexplored when compared to traditional bicyclic β-lactam antibiotics. As reflected by their promising PBP inhibitory activity and resistance against β-lactamase-mediated ring cleavage, valuable structure–activity relationship insights in the monocyclic β-lactam arsenal have been provided, while a contribution has been made to the uphill antibiotics research and development.

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Conflict of interest

The authors declare no conflict of interest.

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