










 Cite this: *RSC Adv.*, 2025, **15**, 26048

Synthesis of L-β-(6-azulenyl)alanine and the fluorescent actin disruptor (6-azuleno)chalasin H†

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Received 2nd July 2025

Accepted 9th July 2025

DOI: 10.1039/d5ra04702a

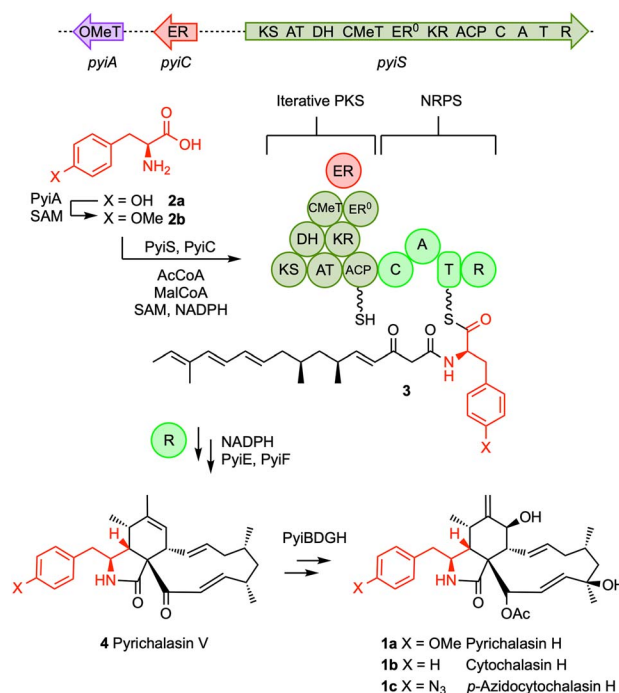
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L-β-(6-Azulenyl)alanine was synthesised for the first time. Supplementation of this compound to *Pyricularia grisea* Δ*pyiA* led to the biosynthesis of the unnatural (6-azuleno)chalasin H and its 1'-bromo congener that have unprecedented natural product skeletons and that are both fluorescent and highly cytotoxic, with IC₅₀ of 0.18 μg mL⁻¹ vs. L929 cells *in vitro*. Actin staining showed that both compounds are potent, but partially reversible, actin disruptors.

Cytochalasins, such as pyrichalasin H **1a**, are a large family of fungal specialized metabolites that are known to bind strongly to actin.^{1–3} This activity prevents elongation of actin filaments and gives cytochalasins corresponding potent bioactivities. Pyrichalasin H **1a** is biosynthesised by a hybrid iterative polyketide synthase non-ribosomal peptide synthetase (iPKS-NRPS, Scheme 1) known as PyiS.⁴ This multi-functional enzyme produces a specific β-keto polyketide that is attached to an acyl carrier protein (ACP). The adenylation (A) domain of the PyiS NRPS activates and selects *O*-methyl tyrosine **2b** and covalently links it to the downstream thiolation (T) domain. The polyketide is then transferred to the amine of the amino acid by the condensation (C) domain to create an enzyme-bound acylaminothioester **3**. The thioester is reduced to release an aldehyde as the first enzyme-free intermediate,⁵ and Knoevenagel⁶ and Diels Alder⁷ enzymes then catalyse two cyclisations to afford the distinctive hexahydroisindolone core of the unfunctionalised pyrichalasin V **4**. Finally, tailoring enzymes (PyiBDGH) create the mature pyrichalasin H **1a**.

Other cytochalasins also incorporate aromatic amino acids. For example, cytochalasin H **1b** is formed from phenylalanine, while the related chaetoglobosins (*vide infra*) are built from tryptophan and a longer polyketide. In previous work we prevented the biosynthesis of *O*-methyl tyrosine in the pyrichalasin H **1a** producing fungus *Pyricularia grisea* by knocking out the *pyiA* gene encoding an *O*-methyltransferase (*O*-MeT) from the *pyi* biosynthetic gene cluster (BGC).⁸

In this mutant, endogenous phenylalanine can weakly complement the missing *O*-methyl tyrosine **2b** to produce low titres of cytochalasin H **1b**. But, when supplemented by various *p*-substituted phenylalanines, good titres of the corresponding *p*-substituted cytochalasins can be produced in a process known as mutasynthesis.⁹ For example, *p*-azidophenylalanine leads to good production of *p*-azidocytochalasin H **1c**. These observations indicate that the A-domain of the pyrichalasin NRPS can accept a wide range of *p*-substituted phenylalanines and that



Scheme 1 Biosynthesis and mutasynthesis of cytochalasins.

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 † Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d5ra04702a>

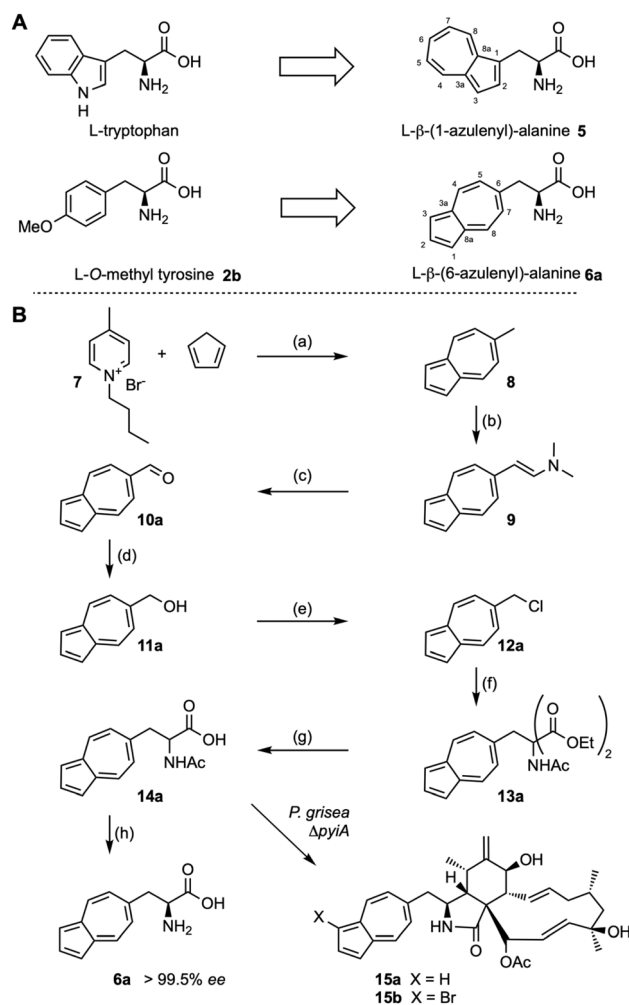

the reductive release (R), cyclisation (PylE, PylF) and late-acting tailoring enzymes (PylBDGH) are unaffected by changes to the amino acid component.

Recent interest in the area of aromatic amino acids has shown that they can often be replaced by corresponding azulenylalanines. Azulene has proven itself to be a useful molecular probe because of its inherent fluorescence.^{10,11} For example, L-β-(1-azulenyl)alanine **5** has been used to replace tryptophan in peptides without significant structural perturbations. Its fluorescence can be quenched by unprotonated histidine, providing a sensitive tool for the measurement of histidine pK_a values in proteins.¹² Kalesse and coworkers have built azulene into the structure of the natural product argyrim.¹³

L-β-(1-Azulenyl)alanine **5** is a carbocyclic analog of L-tryptophan. From our experiments in the area of pyrlichalasin H biosynthesis we already know that tryptophan cannot replace *O*-methyl tyrosine **2b** during the biosynthesis of pyrlichalasin H **1a**.⁷ However, we reasoned that the isomeric β-(6-azulenyl)alanine **6a** might mimic *O*-methyl tyrosine **2b** and be incorporated in its place *via* mutasynthesis (Scheme 1A), providing an inherently fluorescent cytochalasin. But, while β-(1-azulenyl)alanine **5** is known,¹⁴ β-(6-azulenyl)alanine **6a** has not been reported. We therefore set out to synthesise this unusual amino acid and determine if it can be used to produce new members of the cytochalasin family.

The synthesis starts with reaction of *N*-butyl-4-methyl pyridinium bromide **7** with cyclopentadiene under basic conditions with microwave irradiation to give the known 6-methylazulene **8** (Scheme 2B). Treatment of **8** with *N,N*-dimethylformamide dimethylacetal (DMF-DMA) in refluxing DMF then afforded the known enamine **9**. Oxidative cleavage to the aldehyde **10a** (ref. 15) was followed by borohydride reduction to the corresponding primary alcohol **11a**. Treatment of **11a** with tosylchloride in the presence of Et₃N afforded the chloride **12a** rather than the expected tosylate, but **12a** reacted in the next step without problem. Thus, **12a** was treated with the sodium salt of diethyl acetamidomalonate¹⁶ in DMF to afford the fully protected amino acid **13a**. Ester hydrolysis and decarboxylation then gave the racemic *N*-acetyl amino acid **14a**. Finally, treatment of **14a** with porcine kidney acylase in aqueous buffer at 37 °C for 3 days gave the free amino acid **6a**. Marfey's analysis showed this to be the expected L-enantiomer formed in >99.5% enantiomeric excess (see ESI, Fig. S24†).

We cultivated *P. grisea* Δ*pyiA* in liquid media for 7 days. Pulsed supplementation of the culture with **6a** over the first 4 days was followed by a further 3 days of growth. Extraction of the fermentation broth with EtOAc gave an extract that was analysed by LCMS. This showed the presence of a new compound corresponding to **15a** (calc. for [C₃₄H₄₁NO₅]⁺H⁺ 544.3063, obs. 544.3050) with the distinctive UV absorption spectrum of azulene at 278 and 284 nm, albeit in low titre. Repeat of this experiment using the azulenylalanine *N*-acetate **14a** led to better titres of the new compound (Scheme 1B and see ESI Fig. S25–S29† for details). However, feeding with the fully protected diester **13a** was unsuccessful. Purification of the new compound was achieved using mass-directed HPLC-



Scheme 2 (A) Comparison of structures of tryptophan and *O*-methyl tyrosine with L-β-(1- and L-β-(6-azulenyl)alanine respectively; (B) synthesis of L-β-(6-azulenyl)alanine and azulenochalasin. Reagents and conditions: (a) NaH, DMF, microwave, 200 °C, 15 min, 76%; (b) DMF-DMA, DMF, 140 °C, 7 h; (c) NaIO₄, THF/H₂O, RT, 1 h, 69% over two steps; (d) NaBH₄, CH₂Cl₂/MeOH, 0 °C, 30 min, 93%; (e) TsCl, Et₃N, CH₂Cl₂, RT, 16 h; (f) diethyl acetamidomalonate, NaH, DMF, RT, 4 h, 67% over two steps; (g) LiOH·H₂O, THF/H₂O, 50 °C, 2 d, 93%; (h) acylase, phosphate buffer, 37 °C, 3 d, 41%.

fractionation from the feeding experiment with **14a** to give 1.3 mg L⁻¹ of **15a** as a blue solid.

Full characterisation of **15a** by NMR confirmed the expected structure as (6-azuleno)chalasin H. In particular, the ¹H NMR displayed almost identical resonances for the macrolide and hexahydroisindolone core as pyrlichalasin H **1a**, but the distinctive seven aromatic protons of azulene between 7.0 and 8.5 ppm replaced the *p*-methoxyphenyl signals of **1a** (Fig. S39†).

The compound showed a typical maximum UV absorption of 340 nm and fluorescence emission at 380 nm. In an attempt to increase the titre of **15a**, the fermentation was repeated using baffled flasks that have the effect of improving oxygenation and general fungal growth characteristics. Surprisingly, under these conditions, a new compound **15b** was observed (see ESI Fig. S30–S32† for details). The mass of the new compound corresponds to a single bromination of **15a**, (calc. for



$[C_{34}H_{40}^{79}BrNO_5-H]^-$ 620.2012, obs. 620.1989) and this was confirmed by 1H NMR and MS analysis of the purified compound.

Bromination was determined at the 1'-position of azulene by NMR, and consistent with an observed shift in the UV spectrum from 278 and 284 nm to 284 and 295 nm; and fluorescence absorption and emission shifts to 350 nm and 390 nm, respectively (see ESI† for details).¹⁷

Encouraged by these results, we attempted to broaden the synthetic scope. First we synthesised *N*-acetyl β -(6-(1-methyl)azulenyl)alanine **14b** using a parallel synthetic route starting from 1-methylcyclopentadiene (Scheme 3, see ESI† for details). This was supplemented to *P. grisea* Δ *pyiA*, but no incorporation into a new cytochalasin skeleton was observed. In a second experiment we supplemented β -(1-azulenyl)alanine **5** (ref. 13) to a growing culture of *Chaetomium globosum* that produces the tryptophan-derived chaetoglobosin A **16** (Scheme 3).¹⁸ In this case the supplemented β -(1-azulenyl)alanine **5** must compete with endogenous tryptophan for incorporation by the chaetoglobosin PKS-NRPS. LCMS and HRMS analysis of extracts from this experiment showed the clear production of a new compound corresponding to the expected (1-azuleno)globosin A **17** (e.g. calc for $[C_{34}H_{37}NO_5]Na^+$ 562.2569, obs. 562.2594, λ_{max} 278 nm, see ESI Fig. S33–S36† for details). But, purification led to extensive decomposition, and inability to fully characterise this material.

Simulated docking of 6-azulenochalasin H **15a** vs. an actin monomer structure (PDB: 3eku, Fig. 1A) using DiffDock-L¹⁹

showed that azulenchalasin can bind in the barbed end pocket. Purified **15a** and **15b** were tested for cytotoxicity in mouse fibroblast L929 cells. Titration studies showed IC_{50} values of $0.18 \mu g mL^{-1}$ in L929 for both **15a** and **15b**.

Compound **15a** was also tested in KB3.1 cells showing cytotoxicity of $0.029 \mu g mL^{-1}$. Actin disruption activity was tested in human osteosarcoma (U-2OS) cells. Cells were seeded on fibronectin-coated cover slips and treated with low dose ($0.18 \mu g mL^{-1}$) and high-dose ($0.9 \mu g mL^{-1}$) concentrations of respective cytochalasin. After 1 h incubation, cells were fixed with 4% pre-warmed paraformaldehyde and permeabilized using 0.1% Triton X-100. Cellular filamentous actin (F-actin) was stained using Atto488-phalloidin. Azulenchalasin **15a** and its brominated congener **15b** showed significant activity on cellular actin structures, ranging from larger F-actin accumulations up to

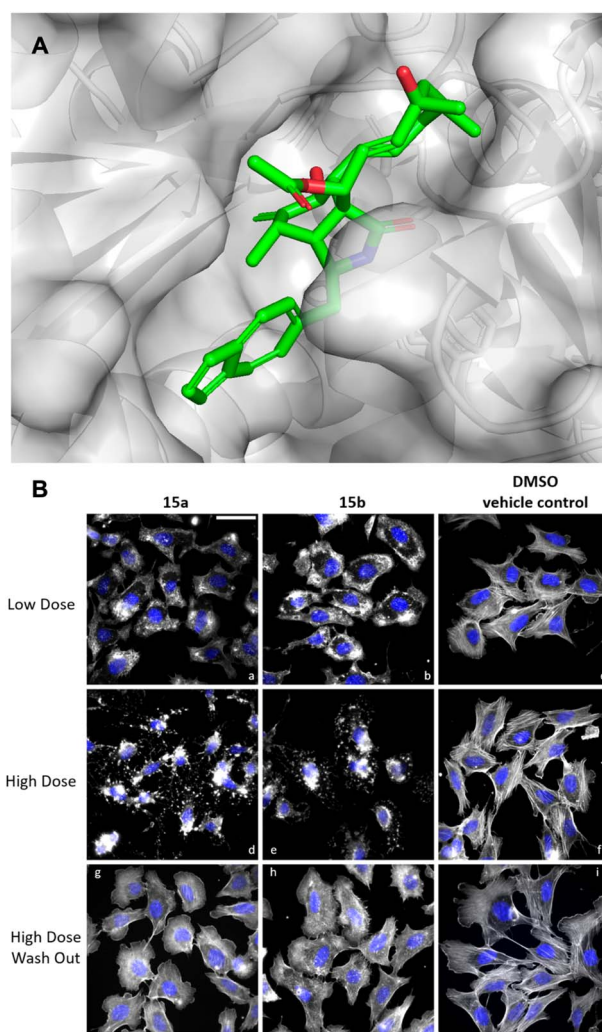
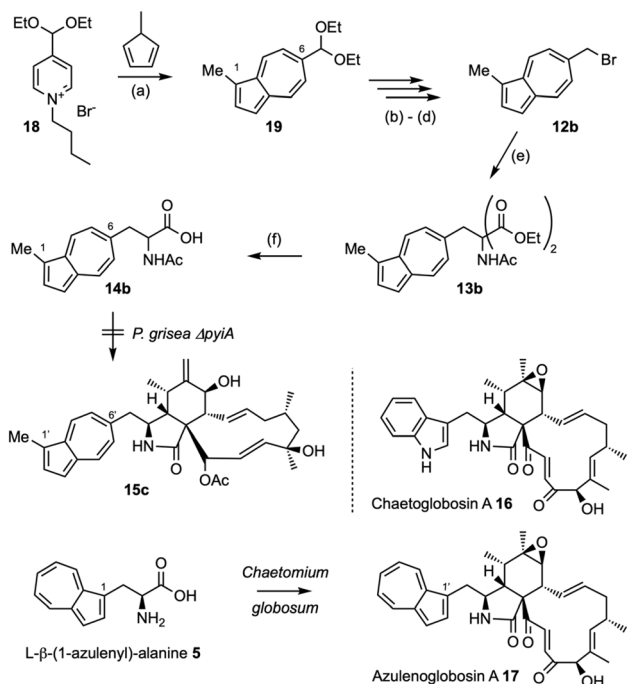


Fig. 1 Biological activity of azulenchalasin. (A) Simulated docking of azulenchalasin **15a** onto an actin monomer (PDB: 3eku) showing binding at the barbed end. (B) *in cellulo* activity of azulenchalasin **15a** and **15b** on the actin cytoskeleton of human osteosarcoma (U-2OS) cells upon low dose ($0.18 \mu g mL^{-1}$) and high dose ($0.9 \mu g mL^{-1}$) treatment as well as high dose treatment followed by wash out and 1 h cultivation in fresh culture medium. Filamentous actin (F-actin) was stained using Atto488-phalloidin (greyscales) and nuclear DNA using DAPI (pseudo coloured in blue). Scale bar in (a) corresponds to $50 \mu m$.



Scheme 3 Synthesis of *N*-acetyl- β -(6-(1-methyl)azulenyl)alanine **14b** and incorporation of *L*- β -(1-azulenyl)alanine **5** into the chaetoglobosin A skeleton. Reagents and conditions: (a) DMF, NaH, 44%; (b) aq. HCl, 81%; (c) $NaBH_4$, MeOH/ CH_2Cl_2 , 92%; (d) CBr_4 , PPh_3 , CH_2Cl_2 ; (e) NaH, DMF, diethyl acetamidomalonate, 42% over two steps; (f) $LiOH \cdot H_2O$, H_2O/THF , 95%.



complete disruption of the network with increasing compound concentration. Notably, the actin disruption observed was not entirely reversible after 1 h recovery period. The cells still lacked pronounced stress fibres after **15a**-treatment, and those previously exposed to **15b** continued to exhibit F-actin accumulation, as well as reduced stress fibres and lamellipodia, as compared to the DMSO control.

Conclusions

Azulenylalanines are inherently fluorescent analogs of aromatic amino acids. Here we have synthesised L-β-(6-azulenyl)alanine **6a** for the first time and in high *ee*. Compound **6a** proves to be an analog of *O*-methyl tyrosine **2b** and is incorporated by the pyrichalasin H synthetase A-domain to create a new cytochalasan skeleton **15a**. Although **6a** was fed to, and incorporated by, *P. grisea*, the *N*-acetate **14a** was a better precursor. Presumably **14a** is more easily imported by *P. grisea* than **6a**, and then hydrolysed *in vivo* to release **6a** as the actual precursor of **15a**. (6-Azulenyl)chhalasin H **15a** itself then appears to be brominated at the 1-position by, most likely, an unknown halogenase to create **15b**.

Remarkably, the new azuleno-chalasin **15a** and **15b** retain the potent actin-disruption properties of native cytochalasins, while possessing inherent fluorescence. The results also indicate that the adenylation (A) domain of the pyrichalasin H PKS-NRPS possesses a remarkable ability to recognise varied aromatic amino acids including azulenes. However, the more-bulky 1-methyl analog, was not incorporated suggesting that the formation of the observed (6-(1-bromo)azuleno)chhalasin H **15b** most-likely arises by adventitious bromination of **15a** itself, rather than by bromination of **6a** before its incorporation by the PylS A-domain. Contrastingly, the chaetoglobosin synthetase is known to accept brominated tryptophan as a substrate during the biosynthesis of chaetoglobosin.¹⁸ Attempts to extend this work to incorporate the tryptophan analog L-β-(1-azulenyl)alanine **5** into chaetoglobosin A was partially successful, also suggesting that the chaetoglobosin A-domain possesses usefully broad substrate selectivity. Detection of the expected compound **17** by LCMS shows that the chaetoglobosin PKS-NRPS A-domain must recognise and activate **5**, but because of competition between **5** and endogenous tryptophan in this experiment, the titre of **17** was very low and insufficient material could be obtained for biological testing or full characterisation. However these results show the remarkable potential of the azulenoalanes as replacements for aromatic amino acids in fungal natural products.

Data availability

The data supporting this article have been included as part of the ESI.†

Author contributions

The project was devised by RJC, JG and MH. RJC, JG, MK and TES supervised the projects. All synthesis and characterisation

work was done by MH, KG, CY and LB. KS performed the biological testing and microscopy. RJC performed the simulated docking. The MS was prepared by RJC and polished and approved by all authors.

Conflicts of interest

RJC is joint editor-in-chief of *RSC Advances*.

Acknowledgements

Funding by DFG is gratefully acknowledged (FOR5170, CO1328/10-1, STR666/10-1 & KA913/28-1). We thank Wera Collisi and Prof. Marc Stadler, HZI Braunschweig, for cytotoxicity testing.

Notes and references

- 1 K. Scherlach, D. Boettger, N. Remme and C. Hertweck, *Nat. Prod. Rep.*, 2010, **27**, 869–886.
- 2 E. Skellam, *Nat. Prod. Rep.*, 2017, **34**, 1252–1263.
- 3 M. A. Tammam, F. Pereira, E. Skellam, S. Bidula, A. Ganesan and A. El-Demerdash, *Nat. Prod. Rep.*, 2025, **42**, 788–841.
- 4 J. Schumann and C. Hertweck, *J. Am. Chem. Soc.*, 2007, **129**, 9564–9565.
- 5 H. Heinemann, H. Zhang and R. J. Cox, *Chem.–Eur. J.*, 2023, e202302590.
- 6 H. Zhang, V. Hantke, P. Bruhnke, E. J. Skellam and R. J. Cox, *Chem.–Eur. J.*, 2021, **27**, 3106–3113.
- 7 V. Hantke, E. J. Skellam and R. J. Cox, *Chem. Commun.*, 2020, **56**, 2925–2928.
- 8 C. Wang, V. Hantke, R. J. Cox and E. Skellam, *Org. Lett.*, 2019, **21**, 4163–4167.
- 9 C. Wang, C. Lambert, M. Hauser, A. Deuschmann, C. Zeilinger, K. Rottner, T. E. B. Stradal, M. Stadler, E. J. Skellam and R. J. Cox, *Chem.–Eur. J.*, 2020, **26**, 13578–13583.
- 10 S. Murata, C. Iwanaga, T. Toda and H. Kokubun, *Chem. Phys. Lett.*, 1972, **13**, 101–104.
- 11 L. C. Murfin and S. E. Lewis, *Molecules*, 2021, **26**, 353.
- 12 P. M. Gosavi, Y. S. Moroz and I. V. Korendovych, *Chem. Commun.*, 2015, **51**, 5347–5350.
- 13 E. Stempel, R. F.-X. Kaml, N. Budisa and M. Kalesse, *Bioorg. Med. Chem.*, 2018, **26**, 5259–5269.
- 14 E. J. Watkins, P. J. Almhjell and F. H. Arnold, *ChemBioChem*, 2020, **21**, 80–83.
- 15 T. O. Leino, M. Baumann, J. Yli-Kauhaluoma, I. R. Baxendale and E. A. A. Wallén, *J. Org. Chem.*, 2015, **80**, 11513–11520.
- 16 N. F. Albertson, *J. Am. Chem. Soc.*, 1946, **68**, 450–453.
- 17 D. Dunlop, L. Ludvíková, A. Banerjee, H. Ottosson and T. Slanina, *J. Am. Chem. Soc.*, 2023, **145**, 21569–21575.
- 18 H. M. Ge, W. Yan, Z. K. Guo, Q. Luo, R. Feng, L. Y. Zang, Y. Shen, R. H. Jiao, Q. Xu and R. X. Tan, *Chem. Commun.*, 2010, **47**, 2321–2323.
- 19 G. Corso, A. Deng, B. Fry, N. Polizzi, R. Barzilay and T. Jaakkola, *arXiv*, 2024, preprint, arXiv:2402.18396v1, DOI: [10.48550/arXiv.2402.18396](https://doi.org/10.48550/arXiv.2402.18396).

