PAPER

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Cross-bridged cyclam derivatives with bis(phosphinate) and phosphinate–phosphonate pendant arms (cb-BPC) as chelators for copper radioisotopes†

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Copper radioisotopes can be used for imaging as well as for therapy and, thus, can form ideal theranostic pairs. The Cu(II) complexes of cross-bridged cyclam (cb-cyclam) derivatives are considered to be highly stable *in vivo*. However, the complexes are mostly formed under harsh conditions not compatible with sensitive biomolecules. Here, a new class of cb-cyclam derivatives, cross-bridged bis(phosphinate) cyclams ("cb-BPC"), were investigated. Ligands with one or two methylene-bis(phosphinate) $-CH_2-PO_2H-CH_2-PO_2H(R)$ (R = H, OH, substituted alkyl) pendant arms were synthesized. Bifunctionalization on the distant phosphorus atom was carried out by employing *P*-nitrobenzyl (R = CH_2-Ph-4-NO_2) precursors and/or, for cb-BPC with two bis(phosphinate) pendant arms, by reactions of silyl-phosphites obtained by silylation of their P(O)–H fragments. The reactive bifunctional groups include amine, carboxylate, azide, isothiocyanate, maleimide and/or tetrazine, and also their orthogonally reactive combination in a single molecule of chelator. The cb-BPCs with one bis(phosphinate) arm were not efficiently radiolabelled with [64]Cu. The cb-BPCs with two pendant arms were radiolabelled even at room temperature and with only a small excess of chelator, leading to a high specific activity. Radiolabelling was fully comparable with that of analogous bis(phosphinate) derivatives of cyclam and identical radiolabelling of cyclam and cb-cyclam derivatives was observed for the first time. The cb-BPCs with two bis(phosphinate) pendant arms represent a new class of rigid chelators for copper radioisotopes that are easily synthetically modifiable, highly hydrophilic and radiolabelled under mild conditions.

Introduction

Personalized medicine has become an indispensable part of the medical arsenal used to identify and cure human diseases. This approach involves the right choice of molecular target

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†Electronic supplementary information (ESI) available: Synthesis of cb-cyclam, compound **C** and phthaloyl-glycine aldehyde, gradients used in chromatographic separations, structure of **bis-1**, ^{31}P NMR spectrum of fully silylated **11**, reduction of nitrile-containing compound **14** to compounds **24** and **24a**, additional radiolabelling data, description of the solid-state structure of compound **C** with X-ray experimental details, and characterization data of the synthesized compounds. CCDC 2341020. For ESI and crystallographic data in CIF or other electronic format see DOI: https://doi.org/10.1039/d4ob01473a

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based on the individual patient biochemistry, conjugation of a drug to a suitable targeting molecule that directs the conjugate to its molecular target, and the diagnostic/therapeutic action of the drug in diseased tissue. Among various ways, the utilization of "theranostic" agents combining diagnostic (imaging) probes and therapeutic drugs is an attractive approach. Nuclear medicine offers the theranostic combination of positron or gamma emitters for diagnostic PET/SPECT imaging with alpha- or beta-emitters useful for therapy. If the imaging and therapeutic radioisotopes are derived from the same element, such one-element theranostic agents are chemically identical and have the same properties *in vivo*.

Radioisotopes of many metal elements have been used in, or have been suggested for, nuclear medicine applications as their features cover a wide range of properties.[1–5] For safe applications, any metal radioisotope requires a suitable chelator, which tightly wraps ions of these elements into thermodynamically stable and, more importantly, kinetically inert complexes.[6] The strong binding of the metal ions ensures the stability of these radiopharmaceuticals *in vivo*, *i.e.*, no unde-

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Org. Biomol. Chem., 2025, **23**, 699–720 | **699**

sired leaching of the metal radioelements from the agents. So-called bifunctional ligands having a reactive group are then used for the conjugation of the complexes to bioactive vectors (*e.g.*, small molecules, (oligo)peptides, antibodies, *etc.*) for targeted delivery. Besides the stability and inertness, the chelators/complexes have to meet some other requirements. For efficient radiolabelling, the ligands should be as selective as possible for the desired metal ion (*i.e.*, radiolabelling is less affected by metal impurities), and complex formation should be very fast. Therefore, the search for suitable chelators is a vital branch of current coordination chemistry.

Among metal elements, copper radioisotopes are promising.[7–9] ^{61}Cu (β^+; $t_{1/2}$ 3.3 h) and ^{64}Cu (17.9% β^+; 39% β^-, $t_{1/2}$ 12.7 h) can be used for PET diagnosis, and ^{64}Cu and mainly ^{67}Cu (β^-; $t_{1/2}$ 61.8 h) are studied as radiotherapeutic isotopes. ^{64}Cu is now available worldwide from various academic and commercial providers. ^{67}Cu recently started to be regularly produced.[10] The last two copper radioisotopes are mainly interesting from the theranostic point of view[11] as they form an "ideal" (or "true", or "matched") metal radioisotope theranostic pair. Several tracers with copper radioisotopes are undergoing clinical trials.[12–14]

Despite a long history of the development of chelators for copper radioisotopes,[4,7–9] the ideal ligand family (fast radiolabelling, kinetic inertness, selectivity for Cu(II) over other metal ions, stabilization of Cu(II) over Cu(I), bifunctionality *etc.*) has not been unambiguously defined. Almost all suggested ligands are derivatives of parent macrocyclic ligands, such as H$_3$nota (tacn derivatives), H$_4$dota (cyclen derivatives), or H$_4$teta (cyclam derivatives); or cryptands, such as sarcophagines (Chart 1). Among them, sarcophagine derivatives are the most suitable ones, forming hexacoordinated Cu(II) complexes fully wrapping the metal ion, and radiolabelled sarcophagine conjugates have entered into several clinical trials.[12]

Ligands used for Cu(II) complexation in early radiopharmaceuticals were based on cyclen or cyclam with fully substituted amine groups, and the complexes have been shown to be insufficiently stable *in vivo*. Currently, the most commonly utilized ligands are derivatives of tacn (1,4,7-triazacyclononane) but the Cu(II) complex of H$_3$nota is not kinetically inert enough.[15] Therefore, other chelators have been sought. As cyclam-based ligands are thermodynamically very selective for divalent copper, a lot of ligands investigated have been based on this macrocyclic skeleton. Complexes of cyclam-based ligands having two coordinating pendant arms were shown to be more kinetically inert and/or stable *in vivo* than those of fully substituted cyclams or H$_3$nota derivatives.[16–20] The most kinetically inert are complexes of compounds derived from cross-bridged cyclam (cb-cyclam).[21–23] However, the diacetic acid derivative (H$_2$cb-te2a, Chart 1) suffers from slow and inefficient radiolabelling.[24] The introduction of phosphonate pendant arm(s) significantly improved the radiolabelling properties of cyclam (BPC ligands, Chart 1)[25,26] as well as cb-cyclam (H$_4$cb-te2p or H$_3$cb-te1a1p, Chart 1)[27–29] derivatives due to better coordination and proton-transfer abilities of the phosphonate groups (if compared to the carboxylate pendants).
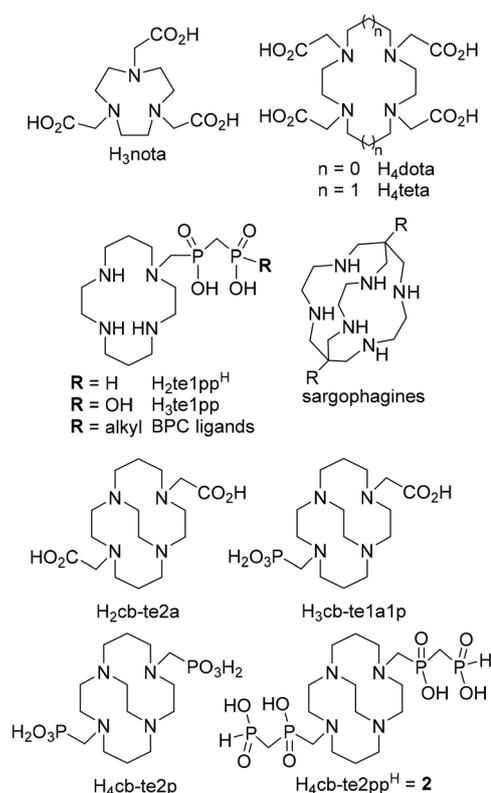

Chart 1 Ligands discussed in the text.

We recently showed that copper(II) binding by cyclam derivatives could be highly improved by the utilization of one geminal methylene-bis(phosphinic acid) or methylene-(phosphinic–phosphonic acid) pendant arm (H$_2$te1pp^H or H$_3$te1pp, respectively; Chart 1).[17] It has also been confirmed for cb-cyclam derivatives H$_4$cb-te2p and H$_4$cb-te2pp^H (Chart 1).[30] Based on these convenient properties of the bis(phosphinate) coordinating unit(s), we introduced a new family of bifunctional ligands, "bis-phosphinate cyclams" (BPC ligands, Chart 1), for the binding of copper radioisotopes.[25,26] The cyclam derivatives exhibit efficacious ^{64}Cu radiolabelling and the radiolabelled tracers are highly stable *in vivo*. Their bifunctionality was introduced onto the distant phosphorus atom of the bis(phosphinic acid) moiety. Modification is far away from the macrocyclic metal binding site and preserves excellent demetallation stability of the complexes, and the conveniently high hydrophilicity of the phosphorus acid containing groups. We have shown that phosphorus atom modification is synthetically accessible, and common bifunctional reactive groups can be used for various coupling reactions.[25,26] A biodistribution study of the radiolabelled BPC conjugate with an antibody against prostate cancer showed much better properties than those with a H$_3$nota derivative.[25]

To date, only a handful of bifunctional cb-cyclam chelators have been proposed. The most commonly used conjugation method is amide coupling through the methylcarboxylate pendant arm (*e.g.*, in H$_3$cb-te1a1p) but it changes the coordi-

nation properties of such ligands, as carboxamides are weakly coordinating groups.[31] A bifunctional reactive group (carboxylate, isothiocyanate) has also been introduced into the cyclam macrocyclic skeleton but this methodology is synthetically inconvenient as it requires the synthesis of the substituted macrocycle.[32,33] In this work, we propose a new class of chelators, "cross-bridged bis(phosphinate) cyclams" (cb-BPC). These chelators offer a flexible approach for the attachment of bifunctional reactive group(s) that utilizes the phosphorus atom(s). It leads to the modification of the most distant phosphorus atom of the bis(phosphinic acid) group(s) and, therefore, the convenient chelation properties of the parent ligand, H_4cb-te2pp^H, are preserved. We also present ^{64}Cu radiolabelling of the ligands.

Results and discussion

Methylene-bis(*H*-phosphinic acids) $CH_2[P(O)(OH)(H)]_2$ (**A**) and (4-NO$_2$-Ph-CH$_2$)(OH)(O)P–CH$_2$–P(O)(OH)(H) (**C**) were obtained by following a published procedure.[25] The solid-state structure of compound **C** was determined by X-ray diffraction (for details, see the ESI†). Methylene-(*H*-phosphinic–phosphonic acid) H_2O_3P–CH$_2$–P(O)(OH)(H) (**B**) was obtained by a partial oxidation of methylene-bis(*H*-phosphinic acid) **A** by HgCl$_2$ followed by the chromatographic removal of unreacted acid **A** and methylene-bis(phosphonic acid). Only very mild oxidation agents such as HgCl$_2$ (suggested for such P–H oxidations previously)[34] could be used to control the oxidation. Utilization of

other common oxidation reagents such as Br$_2$, I$_2$ or peroxo compounds led mainly to methylene-bis(phosphonic acid) or decomposition of the compounds.

Parent cb-BPC chelators

First, we had to find the conditions for the syntheses of the parent bis(*H*-phosphinic acid) derivatives of cb-cyclam. Thus, the simplest derivatives with bis(*H*-phosphinic acid) pendants, **1** and **2** (Scheme 1), were synthesized in a one-step procedure from methylene-bis(*H*-phosphinic acid) **A** and unprotected cb-cyclam using the phospha-Mannich reaction under conditions analogous to those previously reported.[17,25,30] Mono- and di-substitutions were controlled by the reactant stoichiometry. To achieve selectively monosubstituted compound **1**, paraformaldehyde was used as the reaction controlling reagent with an excess of both cb-cyclam and bis(*H*-phosphinic acid) **A**. The non-consumed reactants can be recovered during workup (depending on the purification method). The reaction proceeded well with the products being isolated in ~60% yield on a multigram scale and with only small amounts of side products. The main by-products detected in the reaction mixture were partially oxidized and partially *P*-hydroxymethylated parent acid **A**, *i.e.*, methylene-(*H*-phosphinic–phosphonic) acid **B** and H$_2$O$_2$P–CH$_2$–PO$_2$H–CH$_2$OH, respectively, and a product of double substitution, *i.e.*, compound **2** (~5, ~3, and ~5%, respectively; based on cb-cyclam). The double-substituted compound **2** was prepared previously in moderate yield using an excess of both reactants, bis(*H*-phosphinic acid) **A** and paraformaldehyde, with respect to the amount of cb-cyclam.[30] In
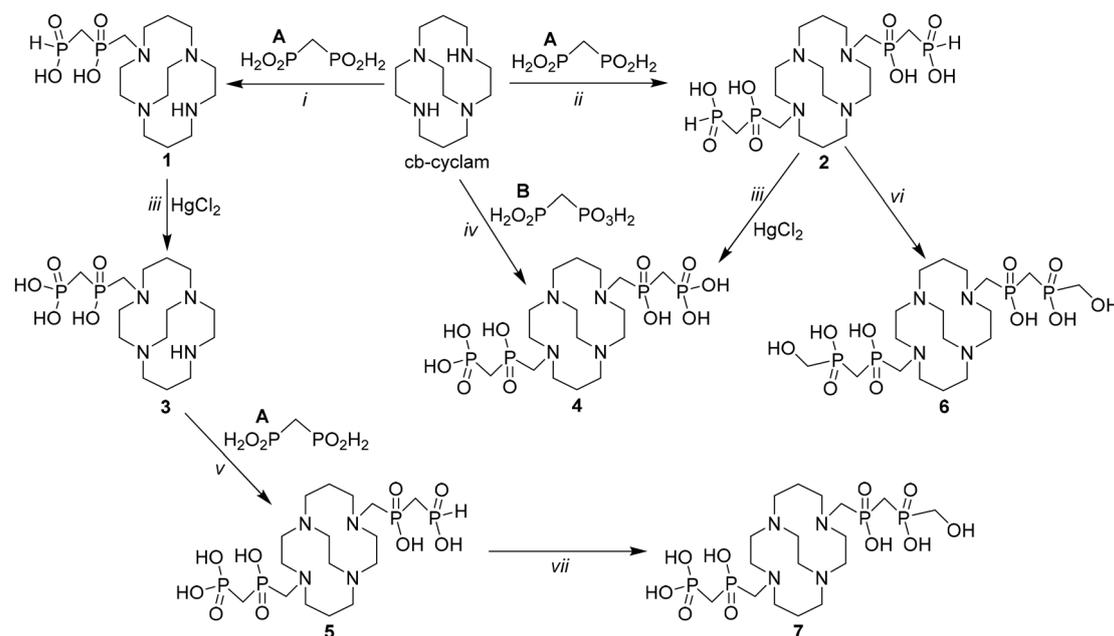
Scheme 1 Syntheses of the parent bis(phosphinic acid) and phosphinic–phosphonic acid derivatives of cb-cyclam. (i) (A, 1 equiv.), paraformaldehyde (0.66 equiv.), 6 M aq. HCl, 60 °C, 2 d (60%, based on formaldehyde). (ii) A (6 equiv.), paraformaldehyde (20 equiv.), 6 M aq. HCl, 60 °C, 2 d (48%).[30] (iii) 1. HgCl$_2$ (1.5 equiv. per P–H group), H$_2$O, 75 °C, 1 d; 2. H$_2$S (>95%). (iv) B (3 equiv.), conc. aq. HCl, paraformaldehyde (2.2 equiv.), 60 °C, 24 h (41%). (v) A (2 equiv.), paraformaldehyde (~1 equiv.), conc. aq. HCl, 60 °C, 3 d (9%). (vi) paraformaldehyde (>30 equiv.), ~90% aq. TFA, 80 °C, 4 d (>90%). (vii) paraformaldehyde (12 equiv.), ~90% aq. TFA, 80 °C, 2 d (>95%).

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Org. Biomol. Chem., 2025, **23**, 699–720 | **701**

attempts to optimize the yield, a lower reactivity of the other secondary amine was observed, leading to the more problematic attachment of the second bis(*H*-phosphinic acid) pendant arm. An interesting by-product was a ditopic ligand, **bis-1** (Fig. S1†), identified by mass and NMR spectra of the reaction mixtures, containing two cb-cyclam fragments **1** bridged by the –CH$_2$–PO$_2$H–CH$_2$–PO$_2$H–CH$_2$– moiety. It probably originates from the reaction of desired product **2** with another molecule of **1** and excess formaldehyde. If an excess of formaldehyde is used in phospha-Mannich reactions, *P*-hydroxymethylation is expected. Here, minimum amounts of *P*-hydroxymethylated derivatives of compounds **1** or **2** as well as only negligible *P*-hydroxymethylation of the starting acid **A** (<5%, see also below) were observed. This points to an unexpected resistance of the geminal bis(*H*-phosphinic acid) fragment towards this side reaction under the conditions used. It contrasts with the reactivity of H$_3$PO$_2$, H$_3$PO$_3$ and various *H*-phosphinic acids; they are readily *P*-hydroxymethylated if they are used as phosphorus components in the phospha-Mannich reactions.[35]

The phosphonic acid pendant arms generally accelerate metal ion complexation and, thus, ligands with a phosphinate–phosphonate group were also prepared. Oxidation of the terminal *H*-phosphinate group(s) of **1** and **2** with HgCl$_2$ analogously to that published[17] led almost quantitatively to derivatives **3** and **4**, respectively, with the geminal phosphinate–phosphonate group(s) (Scheme 1). In the latter case, minor by-products were removed by simple recrystallization. An alternative synthesis of **4** utilizing methylene-(*H*-phosphinic–phosphonic acid) **B** and cb-cyclam was also tested using typical conditions for the phospha-Mannich reaction. The reaction had to be run in a relatively concentrated solution of all components in a closed vial to avoid loss of gaseous formaldehyde. This direct synthesis of **4** using **B** and cb-cyclam did not bring any improvement over the above two-step approach (45% and 41% overall yields in the two- and one-step procedures, respectively).

Unsymmetrically disubstituted cb-cyclam, compound **5**, was obtained in the reaction of compound **3** with methylene-bis(*H*-phosphinic acid) **A** under the same conditions as those used for compound **3** (Scheme 1). However, product **5** was isolated in a very low yield (<10%) as the reaction had to be run with a low conversion of up to only ~15% and with a sub-stoichiometric amount of paraformaldehyde due to easy *P*-hydroxomethylation of compound **5** leading to compound **7** (see also below). It was surprising as *P*-hydroxomethylation was barely observed during the syntheses of compounds **1** and **2**. The use of dilute aq. HCl (1 : 1) instead of conc. aq. HCl significantly suppressed the *P*-hydroxymethylation reaction of compound **5**. In this preparation, the formation of compound **7** had to be prevented as the purification of a mixture of compounds **5** and **7** was not possible.

Aminomethyl-(*H*-phosphinic acids) are generally not fully stable as their P–H bond can spontaneously undergo further reactions such as oxidation in air or reactions with other functional groups in the same molecule. However, the reactivity of the P–H bond can be employed. The simplest way to modify the P–H group is *P*-hydroxymethylation. As given above, some methylene-(*H*-phosphinic acid) moieties react with formaldehyde much less efficiently than the P–H bond in simple *H*-phosphinic acids. This unusually low reactivity of, *e.g.*, methylene-bis(*H*-phosphinic acid) **A** is synthetically useful as phospha-Mannich reactions involving this acid are faster than *P*-hydroxymethylation, and the formaldehyde stoichiometry can be used to control only partial *N*-substitution of azacycles, *e.g.*, in the synthesis of **1**. Commonly, *P*-hydroxymethylation of *H*-phosphinic acid was carried out in hot 1 : 1 aq. HCl with an excess of formaldehyde but these conditions were not satisfactory here (*i.e.*, retro-phospha-Mannich reaction and/or oxidation of *H*-phosphinic acid group(s) were observed). We found that the utilization of hot aq. trifluoroacetic acid (TFA) generally led to better yields. The lower reactivity of the P–H bonds in **1** and **2** with formaldehyde and their "cleaner" phospha-Mannich reactions were further confirmed. Attempts to modify compound **1** with an excess of paraformaldehyde in hot aq. HCl or TFA led to a mixture of compounds where ditopic **bis-1** (Fig. S1†), with a methylene-bis(phosphinic acid) bridge between the cb-cyclam rings of **1**, was detected by MS and ^{31}P NMR; however, isolation or the selective preparation of the compound failed.

The best *P*-hydroxymethylation of compound **2** (to prepare compound **6**) was achieved in ~90% aq. TFA solution at 80 °C (the maximal conversion ~95%) with a high excess of paraformaldehyde (>30 equiv.) added in portions over a period of 4 d. Such harsh conditions led to a slight decomposition of starting compound **2** (mainly oxidation of the terminal *H*-phosphinic acid group(s) to the phosphonic acid group) and purification of this reaction mixture was difficult.

Aqueous TFA can also be used for the efficient preparation of compound **7** in a two-step procedure. In the first step, compound **3** reacted with an excess (2 equiv.) of compound **A** in the phospha-Mannich reaction in aq. HCl to give a mixture of compounds **5** and **7** (see also above). If the reaction was carried out in aq. TFA, it produced a complex mixture. Then, HCl was removed and the residue was dissolved in aq. TFA and more paraformaldehyde was added to produce **7**. A prolonged heating of the mixture of compounds **5** and **7** in aq. HCl led to a partial decomposition of the compounds and to a rich reaction mixture. Due to the easy reaction of compound **5** with formaldehyde in aq. TFA, compound **7** was produced in a good yield (65% based on compound **3**). Thus, we can suggest aq. TFA as a new solvent for the efficient *P*-hydroxymethylation of the P–H bond of terminal *H*-phosphinic acids.

Nitrobenzyl cb-BPC chelators

To obtain bifunctional ligands to utilize these chelators in targeted imaging or therapy, another reactive group(s) has to be introduced onto the ligand skeleton. Ideally, the bifunctional group should be located at a distant position to maintain the coordination properties of the parent ligand. Such a remote bifunctional reactive group can be attached to the geminal bis(phosphinic acid) moiety either through the central methylene

702 | *Org. Biomol. Chem.*, 2025, **23**, 699–720

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carbon or, more conveniently, the distant phosphorus atom. These strategies were recently used for analogous BPC ligands (Chart 1) and it was confirmed that such substitutions did not decrease the radiolabelling efficacy of the BPC ligands.[25]

Compounds with an aryl-nitro group are suitable precursors for bifunctional ligands with the corresponding aryl-amino and aryl-azido groups (Chart 1).[25] Compound **8** (Scheme 2) was obtained by the reaction of *P*-(4-nitro-benzyl) methylene-bis(phosphinic acid) **C**, paraformaldehyde, and cb-cyclam under previously used conditions (6 M aq. HCl, moderate temperature) with a satisfactory yield (62%) as ion exchange purification led to some losses. Several procedures were tested for the preparation of disubstituted derivative **9**. The direct reaction of cb-cyclam and compound **C** led to only a moderate yield (55%, using 6 equiv. of **C**); despite relatively high conversions (>80%), purification was complicated. The utilization of lower excesses of **C** led to significantly lower conversions/yields. Thus, to get **9**, a two-step procedure, with two consecutive phospha-Mannich reactions (with preparation and isolation of **8**), was used; this led to a similar yield (47% over two steps) to that of the direct "one-pot" reaction. The two-step synthesis was easily scaled up and required less compound **C**; however, the procedure overall required more time.

The other secondary amine group in **8** was modified in a reaction with mixed phosphorus acid **B** giving phosphonate-containing ligand **10** in a satisfactory yield (70%; Scheme 2). As the mixed phosphorus acid **B** can be obtained only in a low yield (32%), an alternative route to ligand **10** was tested starting with compound **3** and acid **C** (Scheme 2). The reaction pro-

ceeded in conc. aq. HCl reasonably well, even on a multigram scale (~60% isolated yield). Preparation of compound **11** from mono-substituted cb-cyclam **1** and acid **C** failed as the rich reaction mixture could not be purified (the main impurity was compound **12**). Compound **11** was successfully prepared by the phospha-Mannich reaction of compound **8** with acid **A** in conc. aq. HCl at elevated temperature in a moderate yield (50%; Scheme 2) as, under these conditions, *P*-hydroxymethylation of **11** was not significant (<5%). In these reactions, compounds **1**, **8** and/or **11** do not seem to be fully stable and they undergo a retro-phospha-Mannich reaction with re-formation of a secondary amine and the corresponding *H*-phosphinic acids, and these decomposition products can further react. It was confirmed by isolation of compound **9** (~10%) from the reaction mixture formed in the reaction of **8** to give **11** where acid **C** released from **8** or **11** reacted with still unreacted **8** to produce compound **9**, despite the presence of an excess of acid **A**.

The *P*-hydroxymethylation of acid **C** is much easier than that of acid **A** and this side process decreases the yields of reactions involving acid **C**. The observations point to the fact that the P–H bond in acid **C** is much more reactive than that in acid **A**. The *P*-hydroxymethylation of **11** with an excess of paraformaldehyde easily proceeded in aq. TFA solution to give **12** in a high isolated yield (>90%, Scheme 2) similarly to that for the other disubstituted compounds (see above).

In general, the reactions of compound **8** to give disubstituted derivatives **9**, **10** or **11** proceeded, if compared with the reactions of compound **3**, in relatively high yields. We can
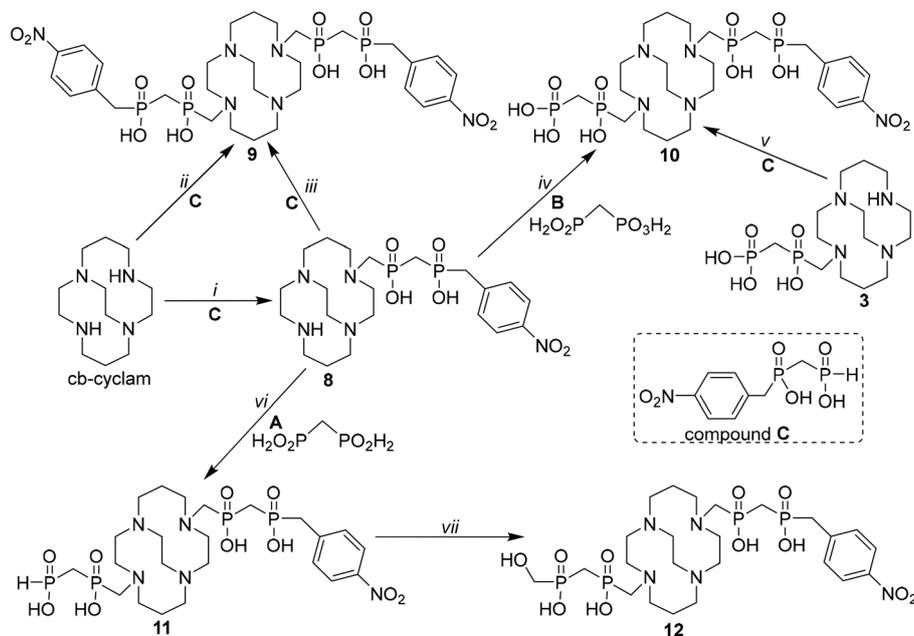
Scheme 2 (i) **C** (1 equiv.), paraformaldehyde (0.66 equiv.), 6 M aq. HCl, 60 °C, 3 d (62%). (ii) **C** (6 equiv.), paraformaldehyde (2.5 equiv.), 12 M aq. HCl, 80 °C, 2 d (55%). (iii) **C** (1.5 equiv.), paraformaldehyde (1.8 equiv.), 12 M aq. HCl, 80 °C, 2 d (76%). (iv) **B** (2.5 equiv.), paraformaldehyde (1.6 equiv.), 12 M aq. HCl, 80 °C, 2 d (70%). (v) **C** (1.5 equiv.), paraformaldehyde (3 equiv.), 12 M aq. HCl, 60 °C, 3 d (65%). (vi) **A** (4 equiv.), paraformaldehyde (3 equiv.), 6 M aq. HCl, 60 °C, 3 d (50%). (vii) Paraformaldehyde (12 equiv.), ~90% aq. TFA, 80 °C, 3 d (>90%).

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Org. Biomol. Chem., 2025, **23**, 699–720 | **703**

speculate that the presence of the bulky and electron-withdrawing nitro-benzyl moiety close to the ring skeleton might alter the conformations and/or intramolecular hydrogen bond system, and the resulting orientation of the remaining secondary amine group is more accessible for the second substitution.
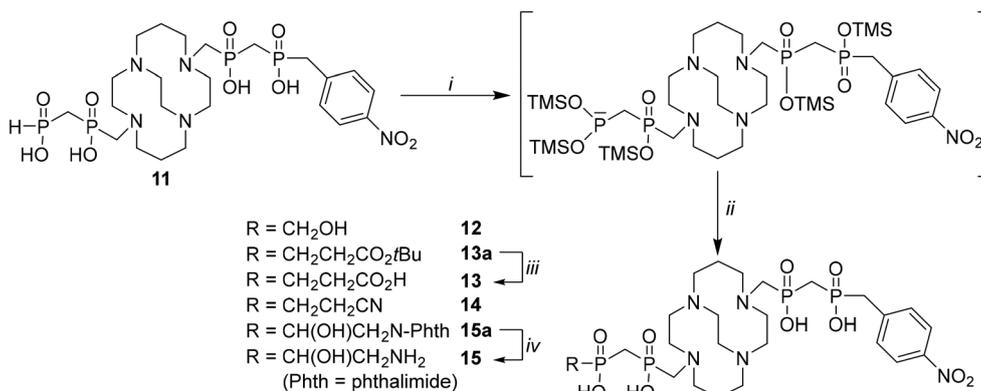
P–H bond modification

Compound **11** could be further modified on the reactive terminal P–H group to add a higher variability on the ligand skeleton. To find the scope of possible reactions, common reactions of the *H*-phosphinic acid group were tested. The five-valent R–P(H)(O)(OH) moiety can be transformed into a very reactive trivalent R–P(OSiMe$_3$)$_2$ group, which can be further transformed.[36,37] Thus, the utilization of a mixture of triethylamine or (*N*,*N*-diisopropyl)-ethylamine (DIPEA), with *N*,*O*-bis(trimethylsilyl)acetamide (BSA) and trimethylsilyl-chloride (TMS-Cl), both used in a large excess, was the most efficient way to transform the R–P(O)(OH)(H) group in **11** into the R–P(OSiMe$_3$)$_2$ moiety almost quantitatively (Scheme 3 and Fig. S2†). Other commonly used silylating agents (*e.g.*, *N*-(trimethylsilyl)imidazole, hexamethyldisilazane, or TMS-Cl alone) with, or without, the amines (DIPEA or Et$_3$N) did not lead to the quantitative formation of the P(III) derivative. Therefore, BSA proved to be necessary in this type of reaction as shown earlier.[38,39,40]

The silylated intermediate derived from **11** was reacted first with activated double bonds,[41,42] such as those in aldehydes or Michael acceptors (Scheme 3). The reactions were carried out with the *in situ* generated intermediate and were quenched by the addition of MeOH to hydrolyse all silyl-containing species. The ligands were extracted into water and, after HPLC purification, compounds **12**, **13a**, **14** and **15a** were obtained in moderate yields (~60% with exception of **14** with 35% yield) and high purity (>95%). The synthesis of compound **12** is less efficient than the direct reaction of **11** with paraformaldehyde in hot aq. TFA (see above) mainly due to purification losses.

The *t*-butyl ester group of compound **13a** was quantitatively cleaved in TFA to give compound **13**, the nitro group of which could be reduced (see below). The phthalimide group of compound **15a** was deprotected with hydrazine in aq. MeOH to obtain compound **15** (70% isolated yield) with an alkyl amine group. Deprotection of compound **15a** in boiling aq. HCl (1 : 1) led to partial decomposition (caused by the retro-phospha-Mannich reaction) and to a lower isolated yield (30%). Another common reaction of phosphites is the Arbuzov reaction with activated alkylhalogenides.[43] Thus, silylated *H*-phosphite **11** was also tested in the reaction with *t*-butyl bromoacetate, benzyl bromide and methyl iodide. These reactions were not selective and mixtures always formed. Besides the desired products, compounds with an alkylated ring amine group were detected and some cleavage of the pendant bis(phosphinic acid) group was also observed by MS after methanolysis of the reaction mixtures. Utilization of an excess of the alkyl halides led to even more complex mixtures. Side reactions significantly decreased the yields and made the purification of the reaction mixtures almost impossible. Thus, only the addition of silylated **11** to activated double bonds can be used to modify the distant *H*-phosphinic acid group. Such reactions can be used for the syntheses of orthogonally bifunctional derivatives of cb-cyclam (see below). It should be noted that silylation was not possible for the parent P–H containing compounds **1**, **2** or **5** as these macrocycles were practically insoluble in any silylation mixture due to their high polarity.
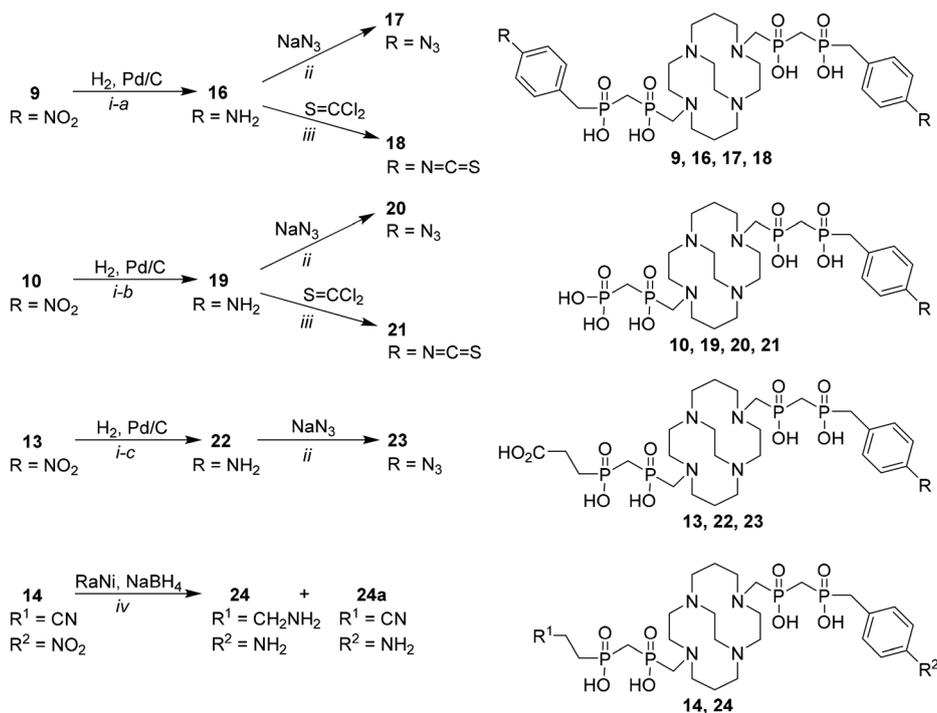
Bifunctional cb-BPC chelators

Ligands with mixed phosphinic–phosphonic acid (*e.g.*, **10**) and those with two bis(phosphinic acid) pendant arms exhibited the best radiolabelling (see below) and, therefore, the synthesis of other bifunctional ligands was exemplified for these two parent motifs. Thus, the 4-nitro group(s) in **9**, **10**, and **13** were reduced by catalytic hydrogenation using Pd/C in water or aq. AcOH to give the corresponding amine group containing ligands **16**, **19**, and **22**, respectively (Scheme 4). The reactions

R = CH$_2$OH **12**
R = CH$_2$CH$_2$CO$_2$*t*Bu **13a**
R = CH$_2$CH$_2$CO$_2$H **13**
R = CH$_2$CH$_2$CN **14**
R = CH(OH)CH$_2$N-Phth **15a**
R = CH(OH)CH$_2$NH$_2$ **15**
(Phth = phthalimide)

Scheme 3 (i) DIPEA (8 equiv.), BSA (16 equiv.), TMS-Cl (3 equiv.), anh. CHCl$_3$, under Ar atm, 50 °C, 1 h (conversion >95% by ^{31}P{^{1}H} NMR). (ii) 1. H$_2$C=CHCO$_2$*t*Bu/H$_2$C=CH–CN/paraformaldehyde/PhtCH$_2$CHO (5 equiv.), anh. CHCl$_3$, Ar atmosphere, 50 °C, overnight. 2. anh. CHCl$_3$/MeOH (excess), Ar atmosphere, room temperature, 30 min (~60% for **12**, **13a**, and **15a**; and 35% for **14**; over two steps based on **11**). (iii) TFA, room temperature, overnight (>95%). (iv) N$_2$H$_4$ hydrate (20 equiv.), 50% aq. MeOH, room temperature, 2 d (70%).
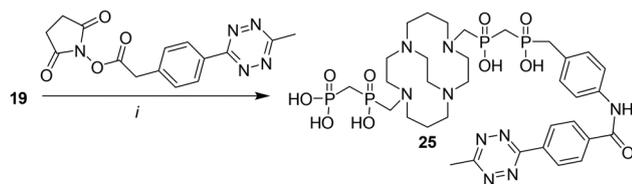
704 | *Org. Biomol. Chem.*, 2025, **23**, 699–720

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Scheme 4 (i-a) Pd/C (20% w/w), H$_2$ (1 atm), ~75% aq. AcOH, 50 °C, 1 d (>95% conversion). (i-b) Pd/C (10% w/w), H$_2$ (1 atm), ~75% aq. AcOH, 50 °C, 2 d (70%). (i-c) Pd/C (20% w/w), H$_2$ (1 atm), ~75% aq. AcOH, 50 °C, 4 h (>95% conversion). (ii) 1. NaNO$_2$ (2.4, 1.5, or 1.5 equiv. for **17**, **20**, and **23**, respectively), 1% aq. HCl, ~0 °C, 5 min; 2. NaN$_3$ (3.5, 2, or 2 equiv. for **17**, **20**, and **23**, respectively), from ~0 °C to room temperature, 3 h (yields: **9 → 17** 57%, **10 → 20** 45%, **11 → 23** 18%). (iii) Thiophosgene (6 and 3 equiv. for **18** and **21**, respectively), 1% aq. HCl/CCl$_4$, room temperature, 24 h (yields: **9 → 18** 55%, **10 → 21** 43%). (iv) Ra–Ni (1 equiv.), NaBH$_4$ (20 equiv.), water, room temperature, 2 d (~10%).

proceeded in aq. AcOH with almost quantitative conversion in a few hours while virtually no reaction was observed in EtOH. Reduction of **10** on a gram scale was performed in ~75% aq. AcOH and ligand **19** was obtained in an isolated yield of 70%. Unfortunately, the anilines are not fully stable upon long-term storage (especially compound **16**, which is also light sensitive) and it is better to use them directly in the next reactions. Therefore, amines **16**, **19** and **22** were converted (Scheme 4) into the corresponding azides **17** and **20**, respectively, through the Sandmeyer reaction by treatment with NaNO$_2$ and NaN$_3$ in aq. HCl with moderate overall yields (~30–50% over two steps, based on the nitro derivatives). The yield of compound **23** (~18%) was determined over four steps as compounds **13a**, **13** and **22** could only be isolated as oils and were always used directly in the next step. Ligand **23**, containing a carboxylic group for amide coupling and an aryl-azide group for alkyne click reactions, represents an orthogonally double bifunctional cb-cyclam-based chelator. The reaction of amines **16** and **19** with thiophosgene under common biphasic conditions (Scheme 4) led to bifunctional ligands **18** and **21**, respectively, with isothiocyanate group(s) in moderate isolated yields (40–50% over two steps based on the nitro derivatives). Reduction of the nitrile group of **14** was problematic. As there is no fully selective reduction of nitriles in the presence of an aryl-nitro group, both groups have to be reduced. However, hydrogenolysis of **14** with Pd/C in aq. AcOH led to a mixture of

compounds without any conversion to the desired product **24**. Commonly used reduction of the nitrile group[44] using freshly prepared Raney nickel was also attempted. Only reactions in alkaline aqueous solutions (**14** is only soluble in water) led to the isolation of compound **24** in a very low yield (~10% on a milligram scale) and the formation of Ni(II) complexes of compounds **24** and **24a** was observed (more details are given in the ESI†). The formation of the Ni(II) complexes in aqueous solution was surprising as, to date, only Cu(II) complexes of cross-bridged cyclams could be prepared in aqueous media. It indirectly proves that methylene-bis(phosphinic acid) pendant arms facilitate the incorporation of metal ions into the cb-cyclam ligand cavity.

To extend the toolbox of bifunctional groups on cb-cyclam bis(phosphinates), a tetrazine moiety for the inverse electron-demand Diels–Alder click reaction was introduced into aniline compound **19** by the reaction with an active ester of a carboxylate tetrazine derivative (Scheme 5). Tetrazine-containing product **25** was obtained in a moderate yield (42%) after semi-preparative HPLC purification. Orthogonally reactive bifunctional ligand **26** (for reactions with thiols and amines) was obtained from the reaction of compound **18** with one equivalent of *N*-(2-aminoethyl)maleimide (Scheme 6). The reaction produced a statistical mixture of di-maleimide **26a** (24%), mono-maleimide **26** (43%) and unreacted di-isothiocyanate **18**. The components of the mixture were separated by semi-pre-

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Org. Biomol. Chem., 2025, **23**, 699–720 | **705**

Scheme 5 (i) NHS-ester of 1,2,4,5-tetrazine derivative (1.1 equiv.), MES–NaOH buffer (1.0 M, pH 6.2, 25 equiv.) in ~50% aq. MeCN, room temperature, 2 d (42%).
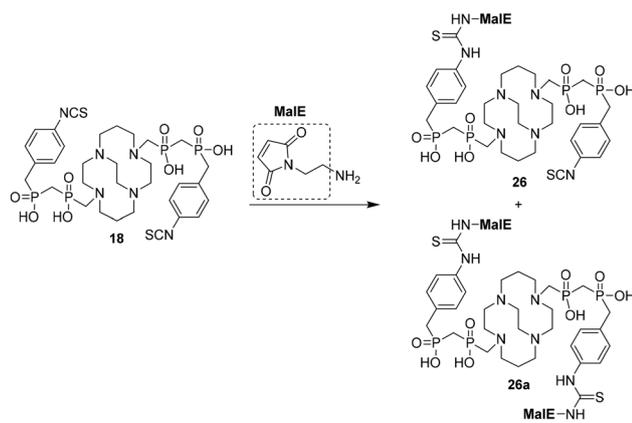
Scheme 6 N-(2-Aminoethyl)maleimide (1 equiv.), DMF, iPr$_2$NEt, room temperature, 5 h, (yields: **26a** 24% and **26** 43%).

parative HPLC after washing of the reaction mixture with AcOEt to remove excess iPr$_2$NEt. These unsymmetrical compounds are the first doubly bifunctional derivatives of cross-bridged cyclam.

Radiolabelling with ⁶⁴Cu

To evaluate the usefulness of the new chelator family for the preparation of copper-based radiopharmaceuticals, the radiolabelling efficiency of the selected ligands was assessed. Radiolabelling was carried out under conditions already used for ⁶⁴Cu radiolabelling of the cyclam-based BPC chelator H$_2$te1pp^H (Chart 1) and its bifunctional P-substituted derivatives/conjugates.[17,25] To compare the title ligands with the well-established chelators H$_3$nota and H$_4$dota and cross-bridged cyclams H$_2$cb-te2a and H$_4$cb-te2p (Chart 1), they were also radiolabelled using the same protocol. The results are shown in Fig. 1. To account for possible differences between various ⁶⁴Cu batches (i.e., to ensure maximum comparability between current and previously obtained data), radiolabelling of H$_2$te1pp^H was always carried out in parallel as a control sample; the ligands were radiolabelled with various ⁶⁴Cu batches and only batches giving the same H$_2$te1pp^H radiolabelling efficacy as that previously published (i.e., radiochemical yield, RCY, ~90 ± 5%)[17,25] were considered (Fig. 1). Radiolabelling was carried out at the pH value commonly used for ⁶⁴Cu radiolabelling (~6.5) and at room temperature. A low molar excess of the chelators with respect to the amount of
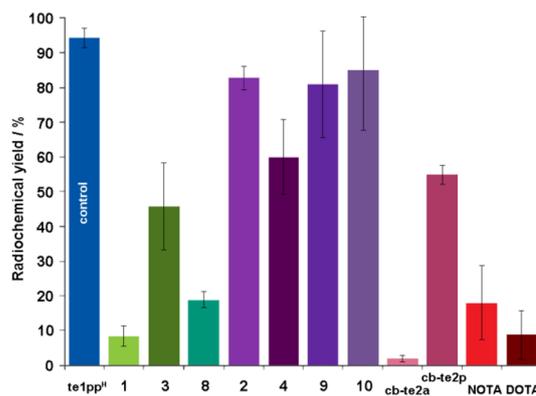

Fig. 1 Comparison of the radiolabelling efficiency of the title chelators with the established chelators H$_3$nota, H$_4$dota, H$_2$cb-te2a, and H$_4$cb-te2p (Chart 1); H$_3$nota data taken from the literature.[17] Data are an average from at least three independent experiments, each done with a freshly prepared batch of 9–11 MBq non-carrier added (NCA) [⁶⁴Cu] CuCl$_2$. Conditions: 0.5 M MES–NaOH buffer, pH 6.2, 25 °C, ~100 equiv. of the chelators with respect to the molar amount of ⁶⁴Cu, labelling time 10 min.

radiocopper (only ~100-times) was used to reach a high specific activity of the radiopharmaceuticals.[17,25]

The results show that H$_3$nota, H$_2$cb-te2a, and ligands **1** and **8** with one bis(phosphinic acid) group were radiolabelled poorly, with a maximum radiochemical yield of less than 25%. The presence of one phosphonic acid group in mono-phosphinic–phosphonic acid derivative **3** significantly improved the radiolabelling yield, if compared with chelators with one bis(phosphinic acid) pendant. The efficacy of ligand **3** is comparable with that of H$_4$cb-te2p with two methylphosphonic acid groups, which has already been suggested as a suitable chelator for ⁶⁴Cu.[27]

Once two bis(phosphinic acid) pendant arms are present in ligands **2**, **9**, or **10**, radiolabelling is further improved and becomes comparable to that of the simple BPC cyclam chelator with one bis(phosphinate) pendant arm (H$_2$te1pp^H, Chart 1).[17] This behaviour is in agreement with our previous study of the complexation mechanism, where ligand **2** was shown to complex Cu(II) at a very high rate, approaching the complexation rate of H$_2$te1pp^H.[30] The comparable radiolabelling of cyclam and cross-bridged cyclam derivatives was observed here for the first time. Similarly to bifunctional BPC,[25] efficient radiolabelling is also preserved for bifunctional cb-BPC. Rather surprisingly, derivative **4** with two phosphinic–phosphonic acid pendants binds ⁶⁴Cu more slowly than other fully substituted derivatives.

The observed radiolabelling behaviour of the chelators could be connected to the mechanism suggested for the complexation of macrocyclic ligands and Cu(II).[17,26,30] In the mechanism, a so-called out-of-cage complex (where only pendant arms are bound to the metal ion and the ring amines are protonated) is formed in the first fast step as a kinetic intermediate (the presence of such kinetic intermediates has been proved in complexation mechanisms for a range of metal

ion–macrocycle systems).[17,26,30,45] The rate-determining step is proton removal from the amine group(s) with simultaneous transfer of the metal ion to the ligand cavity to form the final complex (*in-cage* complexation). Phosphorus acid pendant arms generally assist proton transfer from the ligand cavity to the bulk solvent due to their ability to form hydrogen bonds and their high hydrophilicity.[17,18,26,30] The cb-BPC chelators with two bis(phosphinate) groups are able to "catch" the diluted metal radioisotope to form an *out-of-cage* complex even better than H$_4$cb-te2p and the groups help with Cu(II) transfer to the inner ligand cavity. The *out-of-cage* complexes of chelators with only one bis(phosphinate) group or with carboxylate groups are not thermodynamically stable enough and their radiolabelling is not efficient. On the other hand, an *out-of-cage* complex of ligand **4** with two phosphinate–phosphonate pendants is probably too thermodynamically stable (phosphonates form more stable complexes with metal ions than phosphinates),[46] and it might lead to deceleration of the transfer of the metal ion to the inner ligand cavity and, therefore, to a lower radiochemical yield.

The first protonation of the phosphonate group in macrocyclic complexes takes place at around pH 5.5.[17,18,30] Phosphonate group protonation should lead to a decrease in the stability of *out-of-cage* complexes as well as to a lower ability of the phosphorus acid moiety to transfer proton(s) from the ligand cavity to bulk solution. Both effects should lead to a deceleration of the complexation reaction. To check the effect of phosphonate group protonation on radiolabelling efficiency, radiolabelling was carried out at pH 5.6 (Fig. S3†). As this pH is not optimal for fast radiolabelling, the reaction time had to be increased to 1 h. The results agree with the above assumption. Radiolabelling of the phosphonic acid derivatives was worse than that at pH 6.2. The bis(phosphinic acid) groups are fully deprotonated at pH > 3 and their ability to participate in the above complexation mechanism is not altered as much. Therefore, radiolabelling of the bis(phosphinic acid)-containing ligands is not as sensitive to pH. This observation qualitatively agrees with the pH dependences of the complexation rates observed for the ligands at millimolar reactant concentrations.[17,18,26,30] It also points to another advantage of bis(phosphinate) pendant arms attached to macrocycles – efficient radiolabelling of such chelators might not require such strict pH control.

To find a limit of specific activity for this chelator family, conditions for quantitative labelling were checked. When radiolabelling was carried out at room temperature and pH 6.2, parent derivative **2** with two bis(phosphinate) pendant arms was quantitatively labelled with only ~90 equivalents of the chelator with respect to the amount of [^{64}Cu]CuCl$_2$ (9–11 MBq) in 30 min. It led to a very high molar activity of ~90 GBq per μmol, which was comparable to that of the simple cyclam derivative H$_2$te1pp^H.[17]

The data confirm that the bis(phosphinic acid) group is a suitable moiety to increase the efficiency of metal isotope radiolabelling. They show that even cross-bridged macrocycles can be radiolabelled comparably to common macrocyclic chela-

tors. As only a relatively small excess of the chelator with respect to the amount of radiometal can be used, a very high specific activity is accessible even, with cross-bridged cyclam derivatives under mild radiolabelling conditions.

Stability of ^{64}Cu-radiolabeled complexes

The other aspect relevant for possible *in vivo* applications is the stability of radiolabelled complexes. One of protocols for how to evaluate the *in vitro* stability of the ^{64}Cu-labelled complexes is based on the determination of the extent of ^{64}Cu transchelation to human erythrocyte superoxide dismutase (SOD).[47] Complexes of several ligands prepared here, and of some other ligands (for comparison), were assessed by the method and the results are shown in Fig. 2. To ensure the quantitative incorporation of ^{64}Cu into the *in-cage* complex with all ligands, a large molar excess of (~6000 equiv.) of the chelators with respect to the amount of [^{64}Cu]CuCl$_2$ was used as well as a long labelling time (~2 h incubation at room temperature; except for H$_2$cb-te2a where heating to 50 °C had to be applied).

It is evident (Fig. 2) that complexes of the cb-cyclam-based ligand with two coordinating pendant arms (*i.e.*, H$_2$cb-te2a, H$_4$cb-te2p, **2**, and **4**) underwent almost no transchelation (~1%), suggesting their very high stability, comparable to that of the ^{64}Cu–H$_3$nota complex, which is now generally accepted as stable *in vivo*.[48] In these complexes, an oxygen atom of each pendant arm is bound to the metal ion, leading to octahedral coordination with CN 6.[27,30,49] In contrast, the stability of complexes of monosubstituted cb-cyclam-based ligands (*i.e.*, **1** and **3**) was significantly lower. This could be explained by the incomplete coordination sphere of their *in-cage* complexes with CN 5. Coordination of the distant phosphinate/phosphonate group is unlikely due to steric constraints. Thus in complexes of the fully substituted ligands H$_4$cb-te2p, **2**, and **4**, the Cu(II) ion is completely wrapped by the ligand donor atoms. Another cause of the *in vivo* instability of copper(II) radiopharmaceuticals is their reduction to monovalent copper. Similarly to the Cu(II)–H$_2$cb-te2a complex, divalent copper should be stabilized in Cu(II)–cb-BPC complexes, compared with complexes of other ligand families. Therefore, only the fully substituted cb-cyclam derivatives are suitable for radiochemical applications.
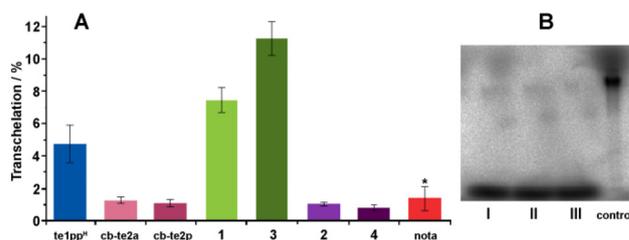
Fig. 2 (A) Transchelation (% of control) of ^{64}Cu complexes of **1**–**4**, H$_2$te2pp^H, H$_2$cb-te2a, H$_4$cb-te2p and H$_3$nota to human erythrocyte superoxide dismutase (SOD). Data for the ^{64}Cu–H$_3$nota complex were taken from the literature.[47] Structures are shown in Chart 1. (B) Example of an audiographic image of the electrophoretic plate (PAGE) obtained for ligand **2** (triplicate, I−III) and the control sample ([^{64}Cu]CuCl$_2$).

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Org. Biomol. Chem., 2025, **23**, 699–720 | **707**

Conclusions

We recently proved that methylene-bis(phosphinic acid) pendant arms accelerated the formation of copper(II) complexes as well as improving the radiolabelling efficiency of cyclam-based chelators. In this work, we extended these observations to cross-bridged cyclam derivatives. Thus, mono- and disubstituted cb-cyclams with simple bis(phosphinic acid) and/or phosphonic–phosphinic acid moieties were synthesized. The phospha-Mannich reaction of methylene-bis(H-phosphinic acid) is significantly faster than its P-hydroxymethylation and, together with a much higher reactivity of the first amine group of cb-cyclam, it enables mono-substitution using formaldehyde as the reaction-controlling reagent and without any protection of the macrocycle. Yields of double-substituted cb-cyclams depend on the structures of both reagents, on H-phosphinic acid and on the type of first substituent on the cb-cyclam skeleton. The P–H bond on the distant phosphorus atom in the disubstituted derivatives can be fully silylated and used in common reactions of trivalent phosphorus; however, only addition reactions to the double bonds are synthetically useful. A range of bifunctional ligands were obtained. The reactive groups involve amino, carboxylic, isothiocyanate, azide, tetrazine and/or maleimide groups, even in an orthogonally reactive combination. Modification on the distant phosphorus atom(s) not participating in metal ion coordination does not alter the chelating/radiolabelling properties of the bifunctional chelators. Radiochemical experiments with ^{64}Cu showed that only one bis(phosphinate) pendant arm was not sufficient for good radiolabelling. Chelators with two bis(phosphinate)/phosphinate–phosphonate pendant arms are radiolabelled very quickly, even at room temperature and with only a small excess of the chelators. The radiolabelling efficiency is similar to that of the cyclam derivatives and such comparably good radiolabelling of cyclams and cb-cyclams has been observed here for the first time. The stability of the radiolabelled cb-cyclams with two bis(phosphinate) pendant arms is the same as that of ^{64}Cu(II) complexes of other disubstituted cb-cyclams. The results presented in this work confirmed that the bis(phosphinic acid) group was a suitable pendant arm to accelerate in-cage complex formation. The data show that the "cross-bridged bis(phosphinate) cyclam" (cb-BPC) derivatives are a novel class of chelators for the development of radiocopper-based radiopharmaceuticals. Their advantages are very efficient radiolabelling, high stability, high hydrophilicity and the location of the reactive group for conjugations far away from the metal binding site, which does not significantly alter radiolabelling and/or the in vivo stability of the complexes.

Experimental section

General

Commercially available (Fluka, Aldrich, CheMatech, Strem) chemicals had synthetic purity and were used as received. Cross-bridged cyclam was purchased as a free base from CheMatech (France) or prepared as the hydrochloride salt by a simplification of the published procedure (details are given in the ESI†).[49] Paraformaldehyde was dried and stored over P_2O_5 in a vacuum desiccator. Methylene-bis(H-phosphinic acid) **A**[50] and (4-nitro-benzyl)(OH)(O)P–CH_2–P(O)(OH)(H) **C**[25] (a simplified procedure is described in the ESI†) were obtained according to published procedures. The single-crystal solid-state structure of **C** was determined (see the ESI† for details). Ligands H_2te1pp^H, H_2cb-te2a, H_4cb-te2p, **2**·$2H_2O$, H_3nota and H_4dota·$2H_2O$ were available from previous studies.[17,30] Syringe filters (PVDF, 0.22 μm pores) were used. The NMR experiments were carried out on Bruker Avance III 600 and 400, Bruker HD850 (^{1}H and ^{13}C{^{1}H}; referenced to external or internal t-BuOH), or Varian S300 (^{31}P and ^{31}P{^{1}H}, referenced to external 85% aq. H_3PO_4; ^{19}F, referenced to external CF_3CH_2OH) NMR spectrometers. For the measurements in H_2O, pre-saturation of the solvent signal was used. The NMR peaks were assigned through standard 2D ^{1}H–^{1}H/^{13}C correlation experiments. The interaction constants are given in Hz. Analytical HPLC was performed on the C-18 column (Cortecs C-18, 4.6 × 50 mm, 2.7 μm, flow rate 1.2 mL min^{-1}) or C-8 column (ReproSil Gold, 5 μm, 120 Å, 150 × 4.6 mm, flow rate 1.0 mL min^{-1}) using the gradient elution of H_2O–MeCN, with or without 0.1% TFA additive (Table S1†). Semi-preparative HPLC was performed on the Waters LC Prep 150 system with C-8 or C-18 (both Phenomenex Luna, 10 μm, 100 Å, 250 × 21.2 mm) columns with flow rates of 15 or 12 mL min^{-1}, respectively, using gradient elution (H_2O–MeCN), both with 0.1% TFA additive. Automatic flash chromatography was performed on an Ecom Toy18DAD800 system with C-18 stationary phase (Büchi Sepacore, 25 × 215 mm, 120 g) using gradient elution (0.1% aq. HCl → MeCN, 100:0 → 100:0 → 0:100 → 0:100 over 5, 18, and 3 min, respectively; Table S1†) with a flow rate 50 mL min^{-1}. Standard ESI-MS spectra and analytical HPLC-MS were recorded on a Waters Acquity QDa instrument (ionization with dual orthogonal ESI at atmospheric pressure) with a quadrupolar analyser in the range of m/z 30–1250, with or without a silica gel column (Cortecs C-18, 4.6 × 50 mm, 2.7 μm particle size, dead time of ~0.4 min) using mobile phases (0.1% aq. TFA and 0.1% TFA in MeCN, various gradients; Table S1†). The conversions were determined by ^{31}P NMR and/or by analytical HPLC. If the compounds were isolated as TFA adducts, the presence of TFA was also confirmed by ^{13}C{^{1}H}/^{19}F NMR. Aluminium foils with silica gel 60 F_{254} (Merck) were used for TLC. High-resolution MS spectra (Bruker APEX-Q FT-MS; in the positive/negative modes, electro-spray ionization) and elemental analyses are presented as "found (calc.)". The methodology for the SOD-challenge experiments (human superoxide dismutase and human serum) was adopted from the literature.[47]

Methylene-[(phosphonic)–(H-phosphinic)]acid (B)

A pre-heated solution (60 °C) of $HgCl_2$ (5.90 g, 21.7 mmol, 1.3 equiv.) in water (60 mL) was added to a pre-heated solution (60 °C) of methylene-bis(phosphinic acid) **A** (2.50 g,

708 | Org. Biomol. Chem., 2025, **23**, 699–720

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17.4 mmol) in water (50 mL) and the mixture was stirred at 60 °C for 3 d (conversion to **B** ~45% by ^{31}P NMR). The mixture was cooled to room temperature and the aqueous phase was decanted from precipitated Hg_2Cl_2. The aqueous phase was further filtered *via* a syringe microfilter (0.22 μm) and the filter was washed with water. The filtrate was saturated with H_2S and precipitated HgS was filtered off analogously to that described above and washed with water. The filtrate was evaporated to dryness and the residue was purified by column chromatography (SiO_2, 100 g, *i*PrOH–conc. aq. NH_3–H_2O 7 : 3 : 3; TLC: R_f(**B**) ~0.2). Fractions with the pure product were combined, evaporated to dryness and co-evaporated several times with water to remove excess ammonia. The residue was re-dissolved in water and the solution was filtered through a syringe microfilter (0.22 μm; removal of a silica precipitate). The filtrate was further purified on a cation exchange resin (Dowex 50, 50 mL, H^+-form, water elution) to remove ammonia quantitatively. The eluate was evaporated to dryness and the resulting oil was further dried to a constant weight (vacuum, 40 °C, 2 d). Product **B** was obtained as a waxy solid (875 mg, 32%). NMR (H_2O + LiOH, pD ≥12): ^{1}H δ 1.93 (CH_2, m, 2H), 7.10 (P–H, dm, 1H, $^1J_{HP}$ 526). ^{13}C{^{1}H} δ 34.6 (CH_2, dd, $^1J_{CP}$ 114, $^1J_{CP}$ 78). ^{31}P δ 12.3 (PO_3H_2 td, $^2J_{PH}$ 18, $^2J_{PP}$ 4), 24.9 (HO$_2$P–H, dtd, $^1J_{PH}$ 526, $^2J_{PH}$ 18, $^2J_{PP}$ 4). ESI-MS: (−) 159.0 (159.0, [M − H]$^-$); (+) 161.0 (161.0, [M + H]$^+$). ESI-HR-MS: (−) 158.96154 (158.96177, [CH$_5$O$_5$P$_2$]$^-$). TLC (*i*PrOH–conc. aq. NH_3–H_2O 7 : 3 : 3): R_f ~0.2. HPLC (C-18, M2): R_f ~0.4 min (in dead volume).

Compound 1

Route a, large scale. In a 100 mL round-bottomed flask, cb-cyclam tetrahydrochloride (5.2 g, 14 mmol, 1.5 equiv.) and methylene-bis(*H*-phosphinic acid) **A** (2.0 g, 14 mmol, 1.5 equiv.) were dissolved in aq. 1 : 1 HCl (90 mL). Paraformaldehyde (0.28 g, 9.3 mmol, 1 equiv.) was added in one portion and the flask was quickly closed with a stopper. The mixture was vigorously stirred and heated to 60 °C for 2 d. The solution was concentrated under vacuum and the residue was co-evaporated with water (2 × 20 mL). The oily residue was dissolved in water (10 mL) and poured onto a cation exchanger (Dowex 50, 3 × 15 cm, H^+-form). The column was washed with water to elute pure acid **A** in the early fractions. After a delay (>500 mL), pure fully substituted compound **2** was eluted with water. After water evaporation under vacuum, compound **2** was obtained as a viscous oil (~100 mg, ~5%; its lyophilisation led to a hydroscopic hexahydrate, **2**·6H$_2$O, elemental analysis (calc. for C$_{16}$H$_{38}$N$_4$O$_8$P$_4$·6H$_2$O, M_R 646.5): C 30.3 (29.7), H 6.6 (7.8), N 8.6 (8.7), P 18.4 (19.2)). Next, the column was washed with 10% aq. pyridine to elute pure product **1**. The solvents were removed under vacuum followed by vacuum drying to get a viscous oily residue, which could be directly used in the next reactions. The oil was dissolved in water (250 mL) and the solution was lyophilized to get slightly hydroscopic zwitterionic **1**·4H$_2$O (2.6 g, 61%). Elemental analysis (calc. for C$_{14}$H$_{32}$N$_4$O$_4$P$_2$·4H$_2$O, M_R 454.4): C 37.3 (37.0), H 8.3 (8.9), N 11.8 (12.3), P 12.5 (13.6).

Route b, small scale. In a 4 mL vial, cb-cyclam (117 mg, 0.52 mmol, 2.4 equiv.) and methylene-bis(*H*-phosphinic acid) **A** (63 mg, 0.44 mmol, 2.0 equiv.) were dissolved in aq. 1 : 1 HCl (~3 mL). To the mixture, paraformaldehyde (6.5 mg, 0.22 mmol, 1 equiv.) was added in one portion and the vial was quickly closed with a stopper. The mixture was vigorously stirred and heated to 80 °C overnight. Then, the solution was concentrated under vacuum and co-evaporated with water (2 × 2 mL). The oily residue was dissolved in water (3 mL) and poured onto an anion exchanger (Amberlite IRA 402, ~3 × 20 cm, OH$^-$-form). Unreacted cb-cyclam (in a sufficient purity to be re-used) was eluted off with water and the product was eluted off with 10% aq. AcOH. After solvent evaporation under vacuum, the residue was co-evaporated with 6 M aq. HCl (~5 mL) and then several times with water to remove any remaining AcOH. The oil was dissolved in a minimum amount of aq. HCl (1 : 1), the solution was evaporated to dryness and the residue was dried under vacuum to obtain **1** as a hydroscopic hydrochloride hydrate, 77 mg (**1**·2.5HCl·3H$_2$O, 67%). Elemental analysis (calc. for C$_{14}$H$_{32}$N$_4$O$_4$P$_2$·2.5HCl·3H$_2$O, M_R 527.6): C 31.8 (31.9); H 7.5 (7.7); N 10.6 (10.6). NMR (H_2O + CsOH, pD ~9): ^{1}H δ 1.65–1.75 (CH$_2$–CH_2–CH$_2$, m, 1H), 1.71–1.81 (CH$_2$–CH_2–CH$_2$, m, 1H), 2.02–2.22 (P–CH_2–P, m, 2H), 2.25–2.35 (CH$_2$–CH_2–CH$_2$, m, 1H), 2.35–2.45 (CH$_2$–CH_2–CH$_2$, m, 1H), 2.50–2.62 (*cycle*, m, 2H), 2.59–2.67 (*cycle*, m, 2H), 2.88–2.96 (*cycle*, m, 3H), 3.01–3.09 (*cycle*, m, 2H), 3.10–3.18 (*cycle*, m, 2H), 3.14–3.20 (N–CH_2–P, m, 1H), 3.19–3.25 (*cycle*, m, 1H), 3.26–3.34 (*cycle*, m, 3H), 3.40–3.48 (*cycle*, m, 2H), 3.59–3.65 (*cycle*, m, 1H), 3.71–3.76 (N–CH_2–P, m, 1H), 3.75–3.81 (*cycle*, m, 2H), 7.13 (P–H, d, 1H, $^1J_{HP}$ 533). ^{13}C{^{1}H} δ 18.9 (CH$_2$–CH_2–CH$_2$, s), 20.1 (CH$_2$–CH_2–CH$_2$, s), 36.0 (P–CH_2–P, dd, $^1J_{CP}$ 84, $^1J_{CP}$ 77), 42.2 (*cycle*, s), 47.7 (*cycle*, s), 49.5 (*cycle*, s), 49.9 (*cycle*, s,), 51.7 (*cycle*, d, $^3J_{CP}$ 6), 54.0 (N–CH_2–P, d, $^1J_{CP}$ 91), 54.4 (*cycle*, s,), 56.2 (*cycle*, s), 58.2 (*cycle*, s), 58.4 (*cycle*, s), 59.5 (*cycle*, s). ^{31}P δ 20.6 (P–H, dtd, 1P, $^1J_{PH}$ 533, $^2J_{PH}$ 17, $^2J_{PP}$ 6), 25.7 (P–CH$_2$–N, m, 1P). ESI-MS: (−) 381.2 (381.2, [M − H]$^-$); (+) 383.3 (383.2, [M + H]$^+$), 405.3 (405.2, [M + Na]$^+$). ESI-HR-MS: (−) 381.18246 (381.18260, [C$_{14}$H$_{31}$O$_4$N$_4$P$_2$]$^-$). TLC (*i*PrOH–conc. aq. NH_3–H_2O 7 : 3 : 3): R_f ~0.7. HPLC (C-18, M2): R_f ~1.0 min.

Compound 3

In a 500 mL flask, powdered compound **1**·4H$_2$O (3.4 g, 7.4 mmol) was dissolved in water (200 mL) and the solution was heated to 75 °C. Then, a hot (75 °C) aq. solution (20 mL) of HgCl$_2$ (3.1 g, 11 mmol, 1.5 equiv.) was added in one portion. The mixture was stirred and heated to 75 °C for 1 d. After completion of the reaction (^{31}P NMR, >95% conversion), the mixture was cooled and the liquid phase was decanted from the precipitate. The aqueous phase was further filtered off through a syringe microfilter (0.22 μm). This clear solution was bubbled with H$_2$S for several minutes. Precipitated HgS was removed by filtration through syringe microfilters (0.22 μm; 2–3 filters were necessary). The solution was then evaporated to dryness under vacuum; the residue was co-evaporated with water (2 × 20 mL) and dried to a constant weight under vacuum. The product **3** hydrochloride was isolated as a viscous oil, which could be directly used in subsequent steps.

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Org. Biomol. Chem., 2025, **23**, 699–720 | **709**

The oil was dissolved in 6 M aq. HCl, the solution was evaporation to dryness under vacuum and the solid was dried under vacuum to give a hygroscopic yellowish powder of hydrochloride hydrate of **3** (**3**·7.5HCl·0.5H$_2$O, 4.6 g, 92%). NMR (D$_2$O + CsOD, pD ~10): ^{1}H δ 1.63–1.69 (CH$_2$–C*H$_2$*–CH$_2$, m, 1H), 1.72–1.78 (CH$_2$–C*H$_2$*–CH$_2$, m, 1H), 1.87–1.97 (P–C*H$_2$*–P, m, 2H), 2.16–2.24 (*cycle*, m, 1H), 2.29–2.37 (*cycle*, m, 1H), 2.57–2.63 (*cycle*, m, 2H), 2.68–2.74 (*cycle*, m, 2H), 2.88–2.94 (*cycle*, m, 2H), 2.96–3.05 (*cycle*, m, 6H), 3.01–3.05 (N–C*H$_2$*–P, 1H), 3.12–3.18 (*cycle*, m, 1H), 3.25–3.33 (N–C*H$_2$*–P and *cycle*, m, 4H), 3.75–3.83 (*cycle*, m, 3H), 4.21–4.27 (N–C*H$_2$*–P, m, 1H). ^{13}C{^{1}H} δ 19.5 (CH$_2$–*C*H$_2$–CH$_2$, s), 20.6 (CH$_2$–*C*H$_2$–CH$_2$, s), 33.7 (P–*C*H$_2$–P, dd, $^1J_{CP}$ 115, $^1J_{CP}$ 88), 42.3 (*cycle*, s), 48.75 (*cycle*, s), 50.0 (*cycle*, s), 50.1 (*cycle*, s), 52.3 (*cycle*, d,), 53.2 (*cycle*, s), 53.4 (N–*C*H$_2$–P, d, $^1J_{CP}$ 87), 55.0 (*cycle*, s), 58.7 (*cycle*, s), 59.1 (*cycle*, s), 59.4 (*cycle*, s). ^{31}P{^{1}H} δ 11.3 (H$_2$O$_3$*P*, d, $^2J_{PP}$ 6.3 Hz, 1P), 27.6 (*P*–CH$_2$–N, d, $^2J_{PP}$ 6.3 Hz, 1P). ESI-MS: (+) 399.2 (399.2, [M + H]$^+$), 421.2 (421.2; [M + Na]$^+$), 791.4 (791.4 [2M + H]$^+$). ESI-HR-MS: (−) 397.17722 (397.17752, [C$_{14}$H$_{31}$O$_5$N$_4$P$_2$]$^-$). TLC (*i*PrOH–conc. aq. NH$_3$–H$_2$O 7 : 3 : 3): R_f ~0.2. Elemental analysis (calc. for C$_{14}$H$_{32}$N$_4$O$_5$P$_2$·7.5HCl·0.5H$_2$O, M_R 680.8): C 25.0 (24.7); H 5.8 (6.0); N 8.2 (8.2). HPLC (C-18, M2): R_f ~0.9 min.

Compound 4

Route a, oxidation. In a 20 mL glass vial, **2**·2H$_2$O (86 mg; 155 μmol) was dissolved in water (5 mL). Then, a solution of HgCl$_2$ (175 mg, 645 μmol, 4.2 equiv.) in aq. HCl (2 M, 5 mL) was added and the mixture was stirred at 60 °C for 2 d. After cooling to room temperature, the resulting suspension was saturated with H$_2$S and precipitated HgS was removed by centrifugation. The supernatant was evaporated to dryness, dissolved in aq. HCl (3%, 0.5 mL) and an excess of *i*PrOH (25 mL) was slowly added to cloudiness. The mixture was left to stand overnight to precipitate the product. The mother liquor was carefully decanted from the precipitate and discarded. Water was added to the residue, and the resulting solution was treated with charcoal and filtered through a syringe filter (0.22 μm). The filtrate was evaporated to dryness and co-evaporated several times with water. The residue was re-dissolved in water (20 mL) and subsequently lyophilized. The product was obtained as a fine off-white powder of hydrochloride hydrate, **4**·2.5HCl·H$_2$O (68 mg, 65%). Elemental analysis (calc. for C$_{16}$H$_{38}$N$_4$O$_{10}$P$_4$·2.5HCl·H$_2$O, M_R 679.6): C 28.4 (28.3); H 6.2 (6.3); N 8.3 (8.2).

Route b, phospha-Mannich reaction. In a 4 mL vial, cb-cyclam (free-base form, 132 mg, 583 μmol, 1 equiv.) and compound **B** (280 mg, 1.75 mmol, 3.0 equiv.) were dissolved in conc. aq. HCl (3 mL) and paraformaldehyde (38 mg, 1.27 mmol, 2.2 equiv.) was suspended in the solution. The flask was quickly closed with a stopper and the suspension was stirred at 60 °C for 1 d. After cooling to room temperature, the reaction mixture was evaporated to dryness and the residue was further co-evaporated with water (2 × 5 mL) to remove excess HCl. The residue was purified on a strong cation exchange resin (Dowex 50, 100 mL, H$^+$-form, water elution). Unreacted acid **B** and HCl were eluted in the early

fractions followed by fractions containing the product. The fractions with the pure product were combined, evaporated to dryness, and further co-evaporated several times with water. The residue was dissolved in water (100 mL) and the solution was lyophilized. Product **4**·4.5H$_2$O (155 mg, 41%) was obtained in the zwitterionic form as a fine white foam. Elemental analysis (calc. for C$_{16}$H$_{38}$N$_4$O$_{10}$P$_4$·4.5H$_2$O, M_R 651.5): C 29.6 (29.5); H 6.9 (7.3); N 8.5 (8.6). NMR (D$_2$O + CsOD, pD ≥12): ^{1}H δ 1.66–1.70 (CH$_2$–C*H$_2$*–CH$_2$, m, 2H), 1.79–1.83 (CH$_2$–C*H$_2$*–CH$_2$, m, 2H), 1.92 (P–C*H$_2$*–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 18, 4H), 2.75–3.30 (*cycle* and N–C*H$_2$*–P, m, 22H), 3.63–3.69 (N–C*H$_2$*–P, m, 2H). ^{13}C{^{1}H} δ 24.5 (CH$_2$–*C*H$_2$–CH$_2$, s), 35.1 (P–*C*H$_2$–P, dd, $^1J_{CP}$ 117, $^1J_{CP}$ 75), 52.5 (*cycle*, s), 52.9 (*cycle*, s), 53.2–53.5 (N–*C*H$_2$–P, m), 53.5–53.9 (*cycle*, CH$_2$–N–CH$_2$–P, m), 56.7 (*cycle*, s), 57.0 (*cycle*, s), ^{31}P{^{1}H} δ 12.4 (H$_2$O$_3$*P*, d, 2P, $^2J_{PP}$ 7); 36.7 (*P*–CH$_2$–N, d, 2P, $^2J_{PP}$ 7). ESI-MS: (−) 569.1 (569.1, [M − H]$^-$), 284.1 (284.1, [M − 2H]$^{2-}$); (+) 571.2 (571.2, [M + H]$^+$), 593.2 (593.2, [M + Na]$^+$). ESI-HR-MS: (−) 569.14573 (569.14656, [C$_{16}$H$_{37}$O$_{10}$N$_4$P$_4$]$^-$), 284.06918 (284.06964, [C$_{16}$H$_{36}$O$_{10}$N$_4$P$_4$]$^{2-}$). TLC (*i*PrOH–conc. aq. NH$_3$–H$_2$O 7 : 3 : 3): R_f ~0.1. HPLC (C-18, M2): R_f ~0.6 min.

Compound 5

In 50 mL pear-shaped flask, compound **3**·7.5HCl·0.5H$_2$O (1.0 g, 1.5 mmol, 1 equiv.), paraformaldehyde (45 mg, 1.5 mmol, 1 equiv.) and methylene-bis(*H*-phosphinic acid) **A** (0.44 g, 3.0 mmol, 2 equiv.) were mixed in aq. HCl (1 : 1, 30 mL). The mixture was stirred at 60 °C for 4 d in a tightly closed reaction vessel. Then, volatiles were removed under vacuum and the residue was co-evaporated with water several times. The residue was dissolved in water (5 mL) and poured onto a strong cation exchanger (Dowex 50, 10 × 3 cm, H$^+$-form). The column was eluted with water and the starting methylene-bis(*H*-phosphinic acid) **A** was obtained in the first fractions in synthetic purity (>90%) to be re-used. In the latter aqueous fraction (after a significant delay), compound **5** was eluted. The combined fractions containing pure **5** were lyophilized to yield waxy compound **5** as the zwitterionic hydrate (**5**·3.5H$_2$O, 80 mg, ~10%). The column was finally washed with 10% aq. pyridine and compound **3** (~0.6 g) was regenerated in synthetic purity (>80%) after removal of volatiles under vacuum; the main impurity (determined by NMR) was compound **5**. NMR (H$_2$O, pH 1.2): ^{1}H δ 1.81–2.05 (CH$_2$–C*H$_2$*–CH$_2$, m, 2H), 2.21–2.35 (CH$_2$–C*H$_2$*–CH$_2$, m, 2H), 2.37 (P–C*H$_2$*–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 17, 2H), 2.43 (P–C*H$_2$*–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 18.5, 2H), 2.83–3.12 (*cycle*, m, 1H), 3.00–3.12 (*cycle*, m, 3H), 3.12–3.21 (*cycle*, m, 2H), 3.21–3.32 (*cycle*, m, 2H), 3.35–3.61 (*cycle* and N–C*H$_2$*–P, m, 10H), 3.61–3.81 (*cycle*, m, 4H), 7.14 (P–*H*, d, $^1J_{HP}$ 560, 1H). ^{13}C{^{1}H} δ 20.7 and 20.7 (2× CH$_2$–*C*H$_2$–CH$_2$, bs), 32.3 (P–*C*H$_2$–P, dd, $^1J_{CP}$ 125, $^1J_{CP}$ 84), 35.7 (P–*C*H$_2$–P, pseudo-t, $^1J_{CP}$–$^1J_{CP}$ 80.5), 48.2 (*cycle*, bs), 49.8–50.8 (*cycle*, m), 53.9–54.7 (*cycle* and N–*C*H$_2$–P, m), 54.7–55.8 (*cycle*, m), 56.6–57.9 (*cycle*, m), 57.9–58.8 (*cycle* and N–*C*H$_2$–P, m). ^{31}P δ 16.8 (H$_2$O$_3$*P*, m, 1P), 20.6 (H$_2$O$_2$*P*, dm, 1P, $^1J_{PH}$ 559), 24.3 (N–C–*P*, m, 1P), 25.4 (N–C–*P*, m, 1P). ESI-MS: (−) 553.2 (553.2, [M − H]$^-$); (+) 555.2 (555.2, [M + H]$^+$). ESI-HR-MS: (−) 553.15096 (583.15165, [C$_{16}$H$_{37}$O$_9$N$_4$P$_4$]$^-$), 276.07193 (276.07219,

710 | *Org. Biomol. Chem.*, 2025, **23**, 699–720

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$[C_{16}H_{36}O_9N_4P_4]^{2-}$). Elemental analysis (calc. for $C_{16}H_{38}N_4O_{10}P_4 \cdot 3.5H_2O$, M_R 617.5): C 31.5 (31.1); H 6.7 (7.4); N 8.9 (9.1), P 19.6 (20.1). HPLC (C-18, M2): R_f ~0.6 min.

Compound 6

In a 4 mL glass vial, compound $2 \cdot 6H_2O$ (40 mg, 62 μmol, 1 equiv.) was dissolved in 90% aq. trifluoroacetic acid (TFA, ~20 mL) and heated to 80 °C. Paraformaldehyde (22 mg, 0.74 mmol, 12 equiv.) was added and the flask was quickly closed with a stopper. After the mixture was stirred at 80 °C for 1 d, another portion of paraformaldehyde (22 mg, 0.74 mmol, 12 equiv.) was added, and the same amount (12 equiv.) of paraformaldehyde was added two more times over 2 d (the mixture was stirred at 80 °C for 4 d in total). Volatiles were removed under vacuum and the residue was co-evaporated several times with water to completely remove TFA. The residue was dissolved in water (2 mL) and poured onto a strong cation exchanger (Dowex 50, 5 × 2 cm, H⁺-form). The product was eluted with water after a significant delay. Fractions containing pure product were combined and the solvents were removed under vacuum to yield zwitterionic $6 \cdot 4H_2O$ as a viscous oil (40 mg, yield >90%; purity >95%, the main impurity was compound 7). NMR (D_2O, pD 1.8): ^1H δ 1.94–2.10 (CH_2–CH_2–CH_2, m, 2H), 2.23–2.35 (CH_2–CH_2–CH_2, m, 2H), 2.47–2.53 (P–CH_2–P, m 4H), 2.90–3.07 (cycle, m, 2H), 3.07–3.23 (cycle, m, 6H), 3.23–3.31 (cycle, m, 2H), 3.41–3.62 (cycle and N–CH_2–P, m, 10H), 3.67–3.85 (cycle, m, 4H), 3.88 (P–CH_2–OH, d, $^2J_{HP}$ 5.2, 4H). $^{13}C\{^1H\}$ δ 19.7–20.9 (2× CH_2–CH_2–CH_2, bs), 31.6–33.2 (P–CH_2–P, m), 46.9–48.7 (cycle, m), 49.7–51.1 (cycle, m), 54.4 (cycle, bs), 54.8–57.5 (cycle and N–CH_2–P, m), 57.5–58.9 (cycle, m), 60.8 (P–CH_2–OH, d, $^2J_{HP}$ 113). $^{31}P\{^1H\}$ δ 21.4–26.6 (m, 2P), 36.5–39.0 (m, 2P). ESI-MS: (−) 597.2 (597.2, $[M - H]^-$); (+) 599.2 (599.2, $[M + H]^+$). ESI-HR-MS: (−) 597.17772 (597.17786, $[C_{18}H_{41}O_{10}N_4P_4]^-$), 268.07454 (268.07473, $[C_{18}H_{40}O_{10}N_4P_4]^{2-}$). TLC (iPrOH–conc. aq. NH₃–H_2O 7:3:3): R_f ~0.5. Elemental analysis (calc. for $C_{18}H_{42}N_4O_{10}P_4 \cdot 4H_2O$, M_R 670.5) C 32.3 (32.2); H 7.1 (7.5); N 8.2 (8.4), P 17.8 (18.5). HPLC (C-18, M2): R_f ~0.6 min.

Compound 7

In a 50 mL pear-shaped flask, compound $3 \cdot 7.5HCl \cdot 0.5H_2O$ (0.40 g, 0.59 mmol, 1 equiv.), paraformaldehyde (35 mg, 1.2 mmol, 2 equiv.) and methylene-bis(H-phosphinic acid) A (0.44 g, 3.0 mmol, 1.5 equiv.) were mixed in conc. aq. HCl (30 mL). The mixture was stirred in a tightly closed reaction vessel at 60 °C for 3 d. Then, volatiles were removed under vacuum and the residue was co-evaporated with water several times. The oily residue was dissolved in ~90% aq. TFA (30 mL) and the solution was heated to 80 °C. Paraformaldehyde (53 mg, 1.8 mmol, 3 equiv.) was added, the flask was quickly closed with a stopper and the mixture was stirred at 80 °C for 3 h. Then, another portion of paraformaldehyde (159 mg, 5.3 mmol, 9 equiv.) was added and the mixture was stirred at 80 °C for 1 d. Volatiles were removed under vacuum and the residue was co-evaporated several times with water. The residue was dissolved in water (5 mL) and poured onto a

strong cation exchanger (Dowex 50, 10 × 3 cm, H⁺-form). The column was washed with water. In the latter aqueous fraction (after a significant delay), pure compound 7 was obtained. The fractions with pure 7 were combined and lyophilized to yield zwitterionic $7 \cdot 3H_2O$ as a waxy solid (245 mg, 65% based on compound 3). NMR (D_2O, pD 1.6): ^1H δ 1.95–2.10 (CH_2–CH_2–CH_2, m, 2H), 2.25–2.37 (CH_2–CH_2–CH_2, m, 2H), 2.47 (P–CH_2–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 19, 2H), 2.49–2.55 (P–CH_2–P, m, 2H), 2.93–3.08 (cycle, m, 2H), 3.08–3.24 (cycle, m, 6H), 3.24–3.33 (cycle, m, 2H), 3.43–3.64 (cycle and N–CH_2–P, m, 10H), 3.68–3.86 (cycle, m, 6H), 3.89 (P–CH_2–OH, d, $^2J_{HP}$ 5.2, 2H). $^{13}C\{^1H\}$ δ 20.5 and 20.8 (2× CH_2–CH_2–CH_2, bs), 31.3–33.1 (2× P–CH_2–P, m), 47.9 (cycle, s), 49.6–51.5 (cycle, m), 54.5 (cycle, bs), 54.8–57.8 (cycle and N–CH_2–P, m), 58.4 (cycle, bs), 60.7 (P–CH_2–OH, d, $^1J_{CP}$ 114). $^{31}P\{^1H\}$ δ 16.6–18.0 (H_2O_3P, m, 1P), 20.4–26.7 (m, 2P), 37.5–39.4 (m, 1P). ESI-MS: (−) 583.2 (583.2, $[M - H]^-$); (+) 585.2 (585.2, $[M + H]^+$). ESI-HR-MS: (−) 583.16168 (583.16221, $[C_{17}H_{39}O_{10}N_4P_4]^-$), 291.07734 (291.07747, $[C_{17}H_{38}O_{10}N_4P_4]^{2-}$). TLC (EtOH–conc. aq. NH₃ 1:1): R_f ~0.3. Elemental analysis (calc. for $C_{17}H_{40}N_4O_{10}P_4 \cdot 3H_2O$, M_R 638.5): C 31.8 (32.0); H 6.9 (7.3); N 8.7 (8.8), P 19.0 (19.4). HPLC (C-18, M2): R_f ~0.6 min.

Compound 8

Route a. In a 20 mL glass vial, cb-cyclam (free-base form, 1.12 g, 5 mmol, 1.6 equiv.) and compound $C \cdot 0.5H_2O$ (940 mg, 3.1 mmol, 1 equiv.) were suspended in 6 M aq. HCl (15 mL). Paraformaldehyde (93 mg, 3.1 mmol, 1 equiv.) was added, the vial was quickly tightly closed and the mixture was stirred at 80 °C for 2 d. The reaction mixture was evaporated to dryness and further co-evaporated several times with water to remove excess HCl. The residue was dissolved in water and purified on a strong cation exchange resin (Dowex 50, 5 × 5 cm, ~100 mL, H⁺-form, H_2O → 10% aq. pyridine elution). The pyridine fraction with the crude product was evaporated to dryness and co-evaporated once with water to remove excess pyridine. The residue was further purified by preparative flash chromatography (C-8, M3). Fractions containing pure product were combined and evaporated to dryness. The residue was dissolved in water (250 mL) and the solution was lyophilized to give $8 \cdot TFA \cdot 1.5H_2O$ as a pale-yellow hygroscopic foam (990 mg, 49%, based on C). Elemental analysis (calc. for $C_{21}H_{37}N_5O_6P_2 \cdot TFA \cdot 1.5H_2O$, M_R 658.6): C 41.8 (42.0), H 6.0 (6.3), N 10.6 (10.6).

Route b. In a 100 mL round-bottomed flask, cb-cyclam hydrochloride (2.5 g, 6.7 mmol, 1.5 equiv.), paraformaldehyde (0.14 g, 4.7 mmol, 1 equiv.), and compound $C \cdot 0.5H_2O$ (2.07 g, 6.8 mmol, 1.5 equiv.) were mixed in 6 M aq. HCl (90 mL) and the flask was quickly closed with a stopper. The mixture was stirred at 60 °C for 3 d. The solvents were removed under vacuum and the residue was co-evaporated with water several times. The residue was dissolved in water (5 mL), the solution was poured onto a strong cation exchanger (Dowex 50, 5 × 10 cm, 200 mL, H⁺-form) and the column was consecutively washed with water (100 mL), 20% aq. EtOH (100 mL), 25% aq. AcOH (100 mL) and again with water (100 mL). Finally, the

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Org. Biomol. Chem., 2025, **23**, 699–720 | 711

product was eluted with 10% aq. pyridine. The fractions with pure product were concentrated and the residue was dried under vacuum to a constant weight to give zwitterionic **8·3H$_2$O** as a slightly hydroscopic yellowish oil (2.41 g, 62%, based on **C**), which was stored in a freezer. Elemental analysis (calc. for C$_{21}$H$_{37}$N$_5$O$_6$P$_2$·3H$_2$O, M_R 571.6): C 44.1 (44.1), H 7.3 (7.6), N 11.8 (12.3), P 10.8 (10.8). NMR (D$_2$O, pD ~3): ^{1}H δ 1.66–1.72 (CH$_2$–C*H*$_2$–CH$_2$, m, 1H); 1.73–1.81 (CH$_2$–C*H*$_2$–CH$_2$, m, 1H), 2.04–2.16 (P–C*H*$_2$–P, m, 2H), 2.23–2.31 (cycle, m, 1H), 2.35–2.43 (cycle, m, 1H), 2.55–2.63 (cycle, m, 2H), 2.61–2.67 (cycle, m, 2H), 2.85–3.07 (cycle, m, 5H), 3.07–3.35 (cycle, P–C*H*$_2$–aryl$_1$ and N–C*H*$_2$–P, m, 10H), 3.35–3.41 (cycle, m, 2H), 3.65–3.71 (cycle, m, 1H), 3.74–3.88 (cycle and N–C*H*$_2$–P, m, 3H), 7.49–7.53 (*o*-phenyl, d, 2H, $^2J_{HH}$ 9), 8.17–8.21 (*m*-phenyl, d, 2H, $^2J_{HH}$ 9). ^{13}C {^{1}H} δ 19.0 (CH$_2$–*C*H$_2$–CH$_2$, bs); 20.2 (CH$_2$–*C*H$_2$–CH$_2$, bs); 33.3 (P–*C*H$_2$–P, dd, $^1J_{CP}$ 85, $^1J_{CP}$ 82), 41.1 (P–*C*H$_2$–aryl, dd, $^1J_{CP}$ 86, $^3J_{CP}$ 3); 42.2 (cycle, s); 47.9 (cycle, s); 49.6 (cycle, s); 49.9 (cycle, s); 51.8 (cycle, d, $^3J_{CP}$ 7); 54.1 (cycle, s); 55.2 (P–*C*H$_2$–N, d, $^1J_{CP}$ 90); 58.4 (cycle, s); 58.7 (cycle, s); 59.5 (cycle, s); 124.4 (*m*-phenyl, s); 131.3 (*o*-phenyl, d, $^3J_{CP}$ 5); 143.9 (*ipso*-phenyl, d, $^2J_{CP}$ 8); 146.8 (*p*-phenyl, s). ^{31}P{^{1}H} δ 23.3 (N–CH$_2$–*P*–CH$_2$, d, 1P, $^2J_{PP}$ 11); 31.1 (CH$_2$–*P*–Bn, d, 1P, $^2J_{PP}$ 11). ESI-MS: (−) 516.2 (516.2, [M − H]$^-$); (+) 518.2 (518.2, [M + H]$^+$), 540.2 (540.2, [M + Na]$^+$). ESI-HR-MS: (−) 516.21443 (516.21463, [C$_{25}$H$_{36}$O$_6$N$_5$P$_2$]$^-$); (+) 518.22894 (518.22918, [C$_{25}$H$_{38}$O$_6$N$_5$P$_2$]$^+$). TLC (EtOH–conc. aq. NH$_3$ 4 : 1): R_f ~0.6. HPLC (C-8, M1): R_f ~5.6 min. HPLC (C-18, M2): R_f ~4.0 min.

Compound 9

Route a. In a 4 mL glass vial, cb-cyclam (free-base form, 36 mg, 0.16 mmol, 1 equiv.), compound **C**·0.5H$_2$O (292 mg, 0.96 mmol, 6 equiv.) and paraformaldehyde (12 mg, 0.39 mmol, 2.5 equiv.) were suspended in conc. aq. HCl (2 mL) and the vial was quickly tightly closed. The suspension was stirred at 80 °C for 2 d (conversion ~80%). After cooling to room temperature, the reaction mixture was evaporated to dryness and further co-evaporated several times with water to remove excess HCl. The residue was dissolved in water (5 mL) and purified on semi-preparative HPLC (C-8, M1) in portions. Fractions with the pure product from each HPLC run were pooled, water was added (final volume ~150 mL) and the solution was lyophilized. Product **9·TFA·2H$_2$O** was obtained as a white foam (84 mg, 55%). Elemental analysis (calc. for C$_{30}$H$_{48}$N$_6$O$_{12}$P$_4$·TFA·2H$_2$O, M_R 958.7): C 40.5 (40.1); H 5.3 (5.6); N 8.9 (8.8).

Route b. In a 20 mL glass vial, **8·TFA·1.5H$_2$O** (234 mg, 0.36 mmol, 1 equiv.) and **C**·0.5H$_2$O (164 mg; 0.54 mmol, 1.5 equiv.) were dissolved in conc. aq. HCl (4 mL). Paraformaldehyde (19 mg, 0.63 mmol, 1.8 equiv.) was added and the vial was quickly closed with a stopper. The resulting mixture was stirred at 80 °C for 2 d. The reaction mixture was evaporated under vacuum to dryness. The residue was purified by semi-preparative HPLC (C-8, M1). Fractions with pure product were pooled and lyophilized to give **9·2.5TFA·0.5H$_2$O** (295 mg, 76%). Elemental analysis

(C$_{30}$H$_{48}$N$_6$O$_{12}$P$_4$·2.5TFA·0.5H$_2$O, M_R 1091.2): C 38.2 (38.1); H 4.7 (4.7); N 7.8 (7.6).

Route c, by-product during the preparation of 11. Compound **9** was also isolated during purification of the reaction mixture containing **11** (after phospha-Mannich reaction; see below) by flash chromatography (C-18, M2). After lyophilization of fractions containing pure **9**, the hydrochloride hydrate of compound **9** was isolated as an off-white cottony solid (0.11 g, 9% based on **8·3H$_2$O**). Elemental analysis (calc. for C$_{30}$H$_{48}$N$_6$O$_{12}$P$_4$·1.5HCl·3.5H$_2$O, M_R 926.4): C 38.9 (39.1); H 6.1 (5.8); N 9.1 (8.8); P 13.4 (13.4); Cl 5.7 (5.2). NMR (D$_2$O + CsOD, pD ~11): ^{1}H δ 1.59–1.72 (CH$_2$–C*H*$_2$–CH$_2$, m, 2H), 1.72–1.85 (CH$_2$–C*H*$_2$–CH$_2$, m, 2H), 1.88–2.02 (2× P–C*H*$_2$–P, m, 4H), 2.75–3.16 (cycle and N–C*H*$_2$–P, m, 20H), 3.20–3.28 (cycle, m, 2H), 3.32 (P–C*H*$_2$–C$_{aryl}$, d, $^2J_{HP}$ 17, 4H), 3.58–3.64 (N–C*H*$_2$–P, m, 2H), 7.54–7.58 (*o*-phenyl, d, 4H, $^2J_{HH}$ 9), 8.19–8.23 (*m*-phenyl, d, $^2J_{HH}$ 9, 4H). ^{13}C{^{1}H} δ 24.4 (CH$_2$–*C*H$_2$–CH$_2$, s), 34.8 (P–*C*H$_2$–P, dd, $^1J_{CP}$ 81, $^1J_{CP}$ 73), 40.6 (P–*C*H$_2$–C$_{aryl}$, d, $^1J_{CP}$ 86). 52.5 (cycle, s), 52.9 (cycle, s), 53.9 (cycle, d, $^3J_{CP}$ 7) 54.0 (P–*C*H$_2$–N, d, $^1J_{CP}$ 100), 56.4 (cycle, s), 57.1 (*C*–N–CH$_2$–P, d, $^1J_{CP}$ 3), 124.2 (*m*-phenyl, s), 131.4 (*o*-phenyl, d, $^3J_{CP}$ 5), 144.8 (*ipso*-phenyl, d, $^2J_{CP}$ 9), 146.6 (*p*-phenyl, s). ^{31}P{^{1}H} δ 31.5 (NCH$_2$–*P*–CH$_2$P, d, 2P, $^2J_{PP}$ 7), 32.2 (PCH$_2$–*P*–Bn, d, 2P, $^2J_{PP}$ 7). ESI-MS: (−) 807.2 (807.2, [M − H]$^-$), 403.1 (403.1, [M + 2H]$^{2+}$); (+) 809.2 (809.2, [M + H]$^+$), 831.2 (831.2, [M + Na]$^+$). ESI-HR-MS: (+) 809.23506 (809.23534, [C$_{30}$H$_{49}$O$_{12}$N$_6$P$_4$]$^+$); (−) 807.22048 (807.22079, [C$_{30}$H$_{47}$O$_{12}$N$_6$P$_4$]$^-$). TLC (EtOH–conc. aq. NH$_3$ 4 : 1): R_f ~0.1. HPLC (C-8, M1): R_f ~6.2 min.

Compound 10

Route a. In a 4 mL glass vial, **8·TFA·1.5H$_2$O** (202 mg, 307 µmol, 1.0 equiv.), compound **B** (124 mg, 775 µmol, 2.5 equiv.) and paraformaldehyde (15 mg, 0.50 mmol, 1.6 equiv.) were suspended in conc. aq. HCl (4 mL) and the vial was quickly tightly closed. The resulting suspension was stirred at 80 °C for 2 d (conversion ~90%). After cooling, the reaction mixture was evaporated under vacuum to dryness and the residue was co-evaporated several times with water to remove excess HCl. The residue was purified on a strong cation exchange resin (Dowex 50, ~50 mL, H$^+$-form; elution with water). Fractions containing the crude product were combined and evaporated to dryness. The residue was re-dissolved in water (10 mL) and purified by semi-preparative HPLC (C-8, M1). The fractions with pure product were quantitatively transferred into a 500 mL round-bottomed flask using additional water and the resulting solution (~200 mL) was lyophilized. Product **10·TFA·2H$_2$O** was obtained in the form of a fine white foam (181 mg, 70%). Elemental analysis (calc. for C$_{23}$H$_{43}$N$_5$O$_{11}$P$_4$·TFA·2H$_2$O, M_R 839.6): C 35.9 (35.8); H 5.5 (5.8); N 8.4 (8.3).

Route b. In a 25 mL pear-shaped flask, the hydrochloride of **3** (1.1 g, 2.8 mmol, 1 equiv.) and compound **C** (1.2 g, 4.3 mmol, 1.5 equiv.) were dissolved in conc. aq. HCl (20 mL). Paraformaldehyde (0.25 g, 8.3 mmol, ~3 equiv.) was added in one portion and the flask was quickly closed with a stopper. The mixture was vigorously stirred and heated to 60 °C for 3 d (conversion >90%). After cooling, the solution was evaporated under vacuum and the residue was co-evaporated twice with

712 | *Org. Biomol. Chem.*, 2025, **23**, 699–720

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water (20 mL). The oily residue was dissolved in water (10 mL) and poured onto a strong cation exchanger (Dowex 50, 70 mL, H$^+$-form) and the column was washed with water (100 mL; removal of unreacted **C** and its *P*-hydroxymethylated derivative), 50% aq. EtOH (50 mL), 25% aq. AcOH (50 mL) and water (100 mL). The pure product was eluted with 10% aq. pyridine (250 mL) and the solvents were removed under vacuum to give a viscous oil, which was dried under vacuum to yield the slightly hygroscopic monopyridine salt of the product (**10**·py·3.5H$_2$O, 1.4 g, 60%, the amount of pyridine was also confirmed by ^{1}H NMR). Elemental analysis (calc. for C$_{23}$H$_{43}$N$_5$O$_{11}$P$_4$·py·3.5H$_2$O, M_R 831.6): C 40.2 (40.4); H 6.4 (6.7); N 10.3 (10.1); P 14.7 (14.9). NMR (D$_2$O, pD ~1): ^{1}H δ 1.96–2.08 (CH$_2$–CH_2–CH$_2$, m, 2H), 2.26–2.38 (CH$_2$–CH_2–CH$_2$, m, 2H), 2.32–2.58 (2× P–CH_2–P, m, 4H), 2.79–3.98 (cycle, N–CH_2–P and P–CH_2–aryl, m, 26H), 7.55–7.59 (*o-phenyl*, d, 2H, $^3J_{HH}$ 9); 8.22–8.26 (*m-phenyl*, d, 2H, $^3J_{HH}$ 9). ^{13}C{^{1}H} δ 20.5 (CH$_2$–CH$_2$–CH$_2$, bs), 21.0 (CH$_2$–CH$_2$–CH$_2$, bs), 32.2 (P–CH$_2$–P, dd, $^1J_{CP}$ 124, $^1J_{CP}$ 84), 32.5–34.1 (P–CH$_2$–P, m), 39.3 (P–CH$_2$–aryl, d, $^1J_{CP}$ 89), 48.0 (cycle, s), 48.1 (cycle, s), 49.9 (cycle, s), 50.1–52.1 (cycle, m), 53.6–59.3 (cycle and P–CH$_2$–N, m), 124.5 (*m-phenyl*, d, $^4J_{CP}$ 3), 131.5 (*o-phenyl*, d, $^3J_{CP}$ 6), 141.8 (*ipso-phenyl*, d, $^2J_{CP}$ 9), 147.2 (*p-phenyl*, s). ^{31}P{^{1}H} δ 17.7–18.3 (*P*O$_3$H$_2$, m, 1P), 22.0–26.0 (*P*–CH$_2$–N, m, 1P), 24.7–26.5 (*P*–CH$_2$–N, m, 1P), 38.4–39.6 (*P*–CH$_2$–aryl, m, 1P). ESI-MS: (−) 688.2 (688.2, [M − H]$^−$), 343.6 (343.6, [M − 2H]$^{2−}$); (+) 690.2 (690.2, [M + H]$^+$). ESI-HR-MS: (−) 688.18319 (688.18368, [C$_{23}$H$_{42}$O$_{11}$N$_5$P$_4$]$^−$), 343.58793 (343.58820, [C$_{23}$H$_{41}$O$_{11}$N$_5$P$_4$]$^{2−}$). TLC (*i*PrOH–conc. aq. NH$_3$–H$_2$O 7 : 3 : 3): R_f ~0.3. HPLC (C-8, M1): R_f ~4.9 min.

Compound 11

In a 50 mL round-bottomed glass flask, compound **8**·3H$_2$O (0.70 g, 1.2 mmol, 1 equiv.), methylene-bis(*H*-phosphinic acid) **A** (0.7 g, 7.6 mmol, 4 equiv.) and paraformaldehyde (0.13 g, 4.3 mmol, 3.5 equiv.) were mixed in conc. aq. HCl (40 mL) and the flask was quickly closed with a stopper. The mixture was vigorously stirred at 60 °C for 3 d. Volatiles were removed under vacuum and the residue was co-evaporated several times with water to remove excess HCl. The residue was dissolved in water (5 mL) and poured onto a strong anion exchanger (Dowex 1, 3 × 10 cm bed, OH$^−$-form, 20% aq. AcOH → aq. HCl (1 : 1) elution). Fractions with the crude product were combined, solvents were removed under vacuum and the residue was co-evaporated several times with water. The residue was subjected to flash chromatography (C-18, M2) leading to a partial purification of **11** and giving pure compound **9** in the later fractions (see also above, 0.11 g, 9%). Fractions containing **11** were evaporated under vacuum and the oily residue was dissolved in water (5 mL), and purified by semi-preparative HPLC (C-18, M4). The fractions containing compound **11** (purity over 90%) were combined and lyophilized to give **11**·TFA·H$_2$O as a fluffy solid with sufficient purity (>90%) for the next reactions (0.50 g, 50%); the main impurity was *P*-hydroxymethylated compound **12**, as determined by NMR and MS. NMR (H$_2$O, pH ~1.8): ^{1}H δ 1.92–2.07 (CH$_2$–CH_2–CH$_2$, m, 2H), 2.26–2.37 (CH$_2$–CH_2–CH$_2$, m, 2H), 2.46 (P–CH_2–P,

pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 17, 2H), 2.48 (P–CH_2–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 16, 2H), 2.89–3.06 (cycle, m, 2H), 3.06–3.24 (cycle, m, 6H), 3.24–3.34 (cycle, m, 2H), 3.34–3.62 (cycle and N–CH_2–P, m, 12H), 3.62–3.80 (cycle, m, 4H), 7.23 (P–H, d, $^1J_{HP}$ 568, 1H), 7.54–7.59 (*o-phenyl*, m, 2H), 8.22–8.27 (*m-phenyl*, m, 2H). ^{13}C {^{1}H} δ 20.1–21.4 (2× CH$_2$–CH$_2$–CH$_2$, bs), 32.7 (P–CH$_2$–P–CH$_2$–aryl, pseudo-t, $^1J_{CP}$–$^1J_{CP}$ 82.5), 34.1–35.6 (P–CH$_2$–P–H, m), 39.0 (P–CH$_2$–aryl, d, $^1J_{CP}$ 89), 47.5–48.5 (cycle, bs), 49.0–52.0 (cycle, m), 54.1–56.4 (cycle and P–CH$_2$–N, m), 57.5–59.6 (cycle, m), 124.6 (*m-phenyl*, d, J_{CP} 3.0), 131.6 (*o-phenyl*, d, J_{CP} 4.7), 141.2 (*ipso-phenyl*, d, $^2J_{CP}$ 9.2), 147.3 (*p-phenyl*, d, J_{CP} 4.2). ^{31}P{^{1}H} δ 22.2–22.7 (*P*–H, m, 1P), 22.0–28.0 (*P*–CH$_2$–N, m, 2P), 39.5–40.6 (*P*–CH$_2$–aryl, m, 1P). ESI-MS: (−) 672.2 (672.2, [M − H]$^−$), 335.6 (335.6, [M − 2H]$^{2−}$); (+) 674.2 (674.2, [M + H]$^+$), 696.2 (696.2, [M + Na]$^+$). ESI-HR-MS: (−) 672.18835 (672.18876, [C$_{23}$H$_{42}$N$_5$O$_{10}$P$_4$]$^−$), 335.59052 (335.59074, [C$_{23}$H$_{41}$N$_5$O$_{10}$P$_4$]$^{2−}$). Elemental analysis (calc. for C$_{23}$H$_{43}$N$_5$O$_{10}$P$_4$·TFA·H$_2$O, M_R 806.6): C 37.1 (37.3); H 6.1 (5.8); N 8.9 (8.7); P 15.8 (15.4). HPLC (C-18, M2): R_f ~3.8 min.

Compound 12

In a 4 mL glass vial, **11**·TFA·H$_2$O (0.12 g, 0.15 mmol, 1 equiv.) was dissolved in ~90% aq. TFA (~3 mL) and heated to 80 °C. Paraformaldehyde (26 mg, 0.87 mmol, 6 equiv.) was quickly added and the flask was immediately closed with a stopper. The mixture was stirred at 80 °C for 1 d. Another portion of paraformaldehyde (26 mg, 0.87 mmol, 6 equiv.) was added and the mixture was stirred in the closed vial at 80 °C for another 1 d. The procedure was repeated with another portion of paraformaldehyde (26 mg, 0.87 mmol, 6 equiv.) on the next day. After 3 d, solvents were removed under vacuum and the residue was co-evaporated several times with water to remove the formaldehyde and excess TFA. The oily residue was dissolved in water (~50 mL) and lyophilized to give fibrous off-white solid **12**·1.5TFA·4H$_2$O (0.13 g, >90%, purity >95%, the impurity was assigned to compound **11** by NMR and HPLC). NMR (D$_2$O, pD ~1.7): ^{1}H δ 1.90–2.08 (CH$_2$–CH_2–CH$_2$, m, 2H), 2.20–2.38 (CH$_2$–CH_2–CH$_2$, m, 2H), 2.38 (P–CH_2–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 16, 2H), 2.49 (P–CH_2–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 16, 2H), 2.88–3.04 (cycle, m, 4H), 3.04–3.35 (cycle, m, 8H), 3.35–3.63 (cycle and N–CH_2–P, m, 10H), 3.63–3.81 (cycle, m, 4H), 3.83 (P–CH_2–OH, d, $^2J_{HP}$ 5.2, 2H), 7.54–7.60 (*o-phenyl*, m, 2H), 8.23–8.28 (*m-phenyl*, m, 2H). ^{13}C{^{1}H} δ 19.9–21.2 (2× CH$_2$–CH$_2$–CH$_2$, bs), 31.6–34.1 (2× P–CH$_2$–P, m), 39.4 (P–CH$_2$–aryl, d, $^1J_{CP}$ 88.6), 47.5–48.9 (cycle, m), 49.5–51.3 (cycle, m), 53.9–59.0 (cycle and N–CH$_2$–P, m), 60.8 (P–CH$_2$–OH, d, $^1J_{CP}$ 113), 124.6 (*m-phenyl*, s), 131.5 (*o-phenyl*, d, $^3J_{CP}$ 5.4), 142.0 (*p-phenyl*), 147.2 (*ipso-phenyl*, d, $^2J_{CP}$ 3.7). ^{31}P{^{1}H} δ 18.5–29.8 (m, 2P), 38.8–39.8 (m, 2P). ESI-MS: (−) 702.2 (702.2, [M − H]$^−$), 350.6 (350.6, [M − 2H]$^−$); (+) 704.2 (704.2, [M + H]$^+$). ESI-HR-MS: (−) 702.19849 (702.19933, [C$_{24}$H$_{44}$O$_{11}$N$_5$P$_4$]$^−$), 350.59575 (350.59602, [C$_{24}$H$_{43}$O$_{11}$N$_5$P$_4$]$^{2−}$). Elemental analysis (calc. for C$_{24}$H$_{45}$N$_5$O$_{11}$P$_4$·1.5TFA·4H$_2$O, M_R 946.7): C 33.9 (34.3); H 5.8 (5.8); N 7.6 (7.4); P 13.4 (13.1); F 7.7 (9.0). HPLC (C-18, M2): R_f ~3.8 min.

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Org. Biomol. Chem., 2025, **23**, 699–720 | **713**

General procedure for compounds 12, 14, and 15a

In an argon-flushed 10 mL glass flask, **11**·H₂O·TFA (98 mg, 0.12 mmol, 1 equiv.) was mixed with anhydrous CHCl₃ (~5 mL). Then, DIPEA (~0.2 mL, ~1.0 mmol, 8 equiv.), BSA (~0.5 mL, 2.3 mmol, 16 equiv.) and Me₃Si–Cl (50 μL, 0.4 mmol, 3 equiv.) were successively added *via* syringe. The resulting suspension was stirred under Ar at 50 °C for 1 h until the mixture clarified (conversion to silyl esters >95%, by ^{31}P{^{1}H} NMR). Then, paraformaldehyde, acrylonitrile, or phthalimido-*N*-methylcarbaldehyde, (40 μL, 114 mg or 18 mg, respectively, 0.60 mmol, 5 equiv.) was added and the solution was stirred under an Ar atmosphere at 50 °C overnight. The reaction was quenched with MeOH (0.2 mL), to hydrolyse the silyl esters, and stirred for 5 min. The reaction mixture was extracted with water (3 × 2 mL) to give the crude product in the aqueous phase and the combined aqueous phases were evaporated under vacuum. The oily residue was dissolved in 50% aq. MeOH (3 mL) and the solution was purified by semi-preparative HPLC (C-18; M5). Fractions containing the pure product were combined and lyophilized. The TFA salts of the products (~95% purity as determined by NMR and HPLC) were obtained as hygroscopic solids (68 mg (~60%) of **12**·1.5TFA·4H₂O, 40 mg (35%) of **14**·1.5TFA·3H₂O and 80 mg (60%) of **15a**·1.5TFA·2.5H₂O) which were stored in a freezer (−20 °C).

Characterization data of **12** were identical to those given above.

Compound **14**: NMR (D₂O, pD 1.5): ^{1}H δ 1.89–2.05 (CH₂–C*H₂*–CH₂, m, 2H), 2.20 (P–C*H₂*–CH₂–CN, pseudo-ddq, J_{HP} 18, J_{HP} 15, $^{3}J_{HH}$ 7.5, 2H), 2.25–2.36 (CH₂–C*H₂*–CH₂, m, 2H), 2.43 (P–C*H₂*–P, pseudo-t, $^{2}J_{HP}$–$^{2}J_{HP}$ 16, 2H), 2.45–2.51 (P–C*H₂*–P, m, 2H), 2.73 (P–CH₂–C*H₂*–CN, dt, $^{3}J_{HP}$ 15, $^{3}J_{HH}$ 7.5, 2H), 2.88–3.02 (*cycle*, m, 2H), 3.02–3.13 (*cycle*, m, 4H), 3.13–3.20 (*cycle*, m, 2H), 3.23–3.31 (*cycle*, m, 2H), 3.41–3.59 (*cycle*, N–C*H₂*–P and P–C*H₂*–aryl, m, 12H), 3.61–3.73 (*cycle*, m, 4H), 7.56–7.59 (*o-phenyl*, m, 2H), 8.24–8.27 (*m-phenyl*, m, 2H); ^{13}C{^{1}H} δ 11.1 (P–CH₂–*C*H₂–CN, d, $^{2}J_{CP}$ 3.1), 20.2–21.2 (2× CH₂–*C*H₂–CH₂, bs), 26.7 (P–*C*H₂–CH₂–CN, d, $^{1}J_{CP}$ 97), 33.1 (P–*C*H₂–P, pseudo-t, $^{1}J_{CP}$–$^{1}J_{CP}$ 79), 33.7 (P–*C*H₂–P, pseudo-t, $^{1}J_{CP}$–$^{1}J_{CP}$ 79), 39.3 (P–*C*H₂–C$_{aryl}$, d, $^{1}J_{CP}$ 89), 47.9–48.7 (*cycle*, m), 50.0–50.6 (*cycle*, m), 54.2–55.8 (*cycle* and N–*C*H₂–P, m), 58.0–58.9 (*cycle*, m), 121.4 (*C*N, d, $^{3}J_{CP}$ 13.9), 124.6 (*m-phenyl*, d, J_{CP} 3.0), 131.6 (*o-phenyl*, d, J_{CP} 5.5), 141.7 (*p-phenyl*, bs), 147.3 (*ipso-phenyl*, d, $^{2}J_{CP}$ 4.0); ^{31}P{^{1}H} δ 23.2–29.9 (m, 2P), 41.1 (m, 1P), 41.9 (m, 1P). ESI-MS: (−) 725.2 (725.2 [M − H]⁻); (+) 727.2 (727.2, [M + H]⁺). ESI-HR-MS: (−) 725.21505 (725.21531, [C₂₆H₄₅O₁₀N₆P₄]⁻), 362.10435 (362.10402, [C₂₆H₄₄O₁₀N₆P₄]²⁻). Elemental analysis (calc. for C₂₆H₄₆N₆O₁₀P₄·1.5TFA·3H₂O, M_R 951.7): C 36.7 (36.6); H 5.4 (5.7); N 8.4 (8.8); P 12.9 (13.0); F 9.7 (9.0). HPLC (C-18, M2): R_f ~3.9 min.

Compound **15a**: NMR (D₂O, pD 1.8): ^{1}H δ 1.89–2.10 (CH₂–C*H₂*–CH₂, m, 2H), 2.21–2.37 (CH₂–C*H₂*–CH₂, m, 2H), 2.37–2.45 (P–C*H₂*–P, m, 2H), 2.45–2.65 (P–C*H₂*–P, m, 2H), 2.84–3.24 (*cycle*, m, 8H), 3.24–3.32 (*cycle*, m, 2H), 3.36–3.62 (*cycle* + N–C*H₂*–P + P–C*H₂*–aryl, m, 12H), 3.62–3.78 (*cycle*, m, 4H), 3.84–3.98 (P–CH(OH)–C*H₂*–N, m, 2H), 4.16 and 4.22 (P–

C*H*(OH)–CH₂–N, 2 × ddd, $^{2}J_{HP}$ 11 and 10, $^{3}J_{HH}$ 7.8 and 3.5, $^{3}J_{HH}$ 3.5 and 3.8, respectively; 1H), 7.43–7.51 (*o-phenyl*, m, 2H), 7.77–7.86 (*Pht*, m, 4H), 8.03–8.15 (*m-phenyl*, m, 2H); ^{13}C{^{1}H} δ 19.9–21.4 (2× CH₂–*C*H₂–CH₂, bs), 30.9–32.3 (P–*C*H₂–P, m), 33.1 (P–*C*H₂–P, pseudo-t, $^{1}J_{CP}$–$^{1}J_{CP}$ 83), 39.3 (P–*C*H₂–aryl, d, $^{1}J_{CP}$ 88), 39.5–39.8 (P–CH(OH)–*C*H₂–N, m), 47.3–48.6 (*cycle*, m), 49.5–50.9 (*cycle*, m), 54.2–56.1 (*cycle* and N–*C*H₂–P, m), 57.8–59.1 (*cycle*, m), 68.7 and 69.0 (P–*C*H(OH)–CH₂–N, d, $^{1}J_{CP}$ 113 and 111, respectively), 124.1 (*Pht*, s), 124.3–124.5 (*m-phenyl*, m), 131.4–131.6 (CH–*Pht*, m), 131.8 (*o-phenyl*, d, J_{CP} 8.5), 135.5 (*Phth*, pseudo-d, J_{CP} 3.4), 141.7–142.0 (*p-phenyl*, bs), 146.9 (*ipso-phenyl*, pseudo-dd, J 16, J 3.9), 170.9 (*C*=O, pseudo-d, J 10.7); ^{31}P{^{1}H} δ 18.0–29.5 (bm, 2P), 34.7 and 35.3 (2 × pseudo-s, 1P), 37.3–39.1 (m, 1P). ESI-MS: (−) 861.2 (861.2, [M − H]⁻), 430.1 (430.1, [M − 2H]²⁻), 1761.4 (1761.4, [2M − H]⁻); (+) 863.2 (863.2, [M + H]⁺), 1725.2 (1725.5, [2M + H]⁺). ESI-HR-MS: (−) 861.23121 (861.23135, [C₃₃H₄₉O₁₃N₆P₄]⁻); (+) 863.24573 (863.24591, [C₃₃H₄₉O₁₃N₆P₄]⁺). Elemental analysis (calc. for C₃₃H₅₀N₆O₁₃P₄·1.5TFA·2.5H₂O, M_R 1092.8): C 40.0 (40.1); H 4.8 (5.3); N 7.5 (7.8); P 11.8 (11.5); F 7.6 (7.9). HPLC (C-18, M2): R_f ~4.4 min.

Compound 15

Route a. In a 4 mL vial, compound **15a**·1.5TFA·2.5H₂O (11 mg, 10 μmol, 1 equiv.) was dissolved in 50% aq. MeOH (~2 mL) and hydrazine monohydrate (5.8 μL, 0.10 mmol, 10 equiv.) was added. The solution was stirred at room temperature for 1 d. Afterwards, another portion of hydrazine monohydrate (5.8 μL, 0.10 mmol, 10 equiv.) was added and the solution was stirred for an additional 1 d. Solvents were removed under vacuum and the oily residue was purified by semi-preparative HPLC (C-18, M5). Fractions containing the pure product were combined and lyophilized. The product was obtained as a hygroscopic solid having the composition **15**·2TFA·4H₂O (8.5 mg, 70%; purity >95% as determined by NMR and HPLC), which was stored in a freezer. Elemental analysis (calc. for C₃₃H₅₀N₆O₁₃P₄·2TFA·4H₂O, M_R 1032.7): C 33.6 (33.7); H 5.6 (5.7); N 8.3 (8.1); P 11.6 (12.0); F 11.6 (11.0).

Route b. In a 10 mL pear-shaped flask, compound **15a**·1.5TFA·2.5H₂O (22 mg, 20 μmol, 1 equiv.) was dissolved in 1 : 1 aq. HCl (~3 mL) and the solution was heated to reflux (120 °C) for 1 d. Solvents were removed under reduced pressure and the oily residue was dissolved in water (~5 mL). The aqueous phase was washed with AcOEt (3 × 1 mL), evaporated to dryness under vacuum and the residue was purified by semi-preparative HPLC (C-18, M5). Fractions containing the pure product were combined and lyophilized. The product was obtained as a hygroscopic solid as above (6.5 mg, 30%, purity >95% as determined by NMR and HPLC). NMR (D₂O, pD 2.3): ^{1}H δ 1.82–2.12 (CH₂–C*H₂*–CH₂, m, 2H), 2.17–2.41 (CH₂–C*H₂*–CH₂, m, 2H), 2.31 (P–C*H₂*–P–CH₂–aryl, pseudo-t, $^{2}J_{HP}$–$^{2}J_{HP}$ 16, 2H), 2.33 (P–C*H₂*–P–CH(OH), m, 2H), 2.83–3.23 (*cycle*, m, 9H), 3.23–3.31 (*cycle*, m, 2H), 3.36–3.41 (*cycle*, m, 1H), 3.44 (P–C*H₂*–aryl, d, $^{2}J_{HP}$ 17.8, 2H), 3.41–3.65 (*cycle* and P–C*H₂*–N, m, 10H), 3.65–3.83 (*cycle*, m, 4H), 4.02–4.11 (P–C*H*(OH)–CH₂, m, 1H), 7.54–7.58 (*o-phenyl*, m, 2H), 8.22–8.26 (*m-phenyl*, m, 2H); ^{13}C

714 | *Org. Biomol. Chem.*, 2025, **23**, 699–720

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{^{1}H} δ 19.8–21.1 (2× CH$_2$–CH$_2$–CH$_2$, bs), 33.2 (P–CH$_2$–P–CH$_2$–aryl, pseudo-t, $^1J_{CP}$–$^1J_{CP}$ 79), 33.4–34.2 (P–CH$_2$–P–CH(OH)–CH$_2$, m), 39.7 (P–CH$_2$–aryl, dd, $^1J_{CP}$ 88, $^3J_{CP}$ 5.6), 41.0–41.2 (P–CH(OH)–CH$_2$–N, m), 47.8–48.7 (cycle, m), 49.6–50.9 (cycle, m), 53.8–55.1 (cycle and N–CH$_2$–P, m), 57.6–58.8 (cycle, m), 124.5 (m-phenyl, d, J_{CP} 3.0), 131.5 (o-phenyl, d, J_{CP} 5.2), 142.7 (p-phenyl, bs), 147.1 (ipso-phenyl, d, $^2J_{CP}$ 3.8); ^{31}P{^{1}H} δ 21.4–24.0 (m, 1P), 24.0–26.3 (m, 1P), 29.9–31.5 (m, 1P), 34.2–36.7 (m, 1P). ESI-MS: (−) 731.2 (731.2, [M − H]$^−$), 365.1 (365.1, [M − 2H]$^{2−}$), 1463.5 (1463.5, [2M − H]$^−$); (+) 733.2 (733.2, [M + H]$^+$). ESI-HR-MS: (−) 731.22600 (731.22588, [C$_{25}$H$_{47}$O$_{11}$N$_6$P$_4$]$^−$), 365.10934 (365.10930, [C$_{25}$H$_{46}$O$_{11}$N$_6$P$_4$]$^{2−}$); (+) 733.23995 (733.24043, [C$_{25}$H$_{47}$O$_{11}$N$_6$P$_4$]$^+$). HPLC (C-18, M2): R_f ~3.8 min.

Compound 13

In an argon-flushed 4 mL glass vial, **11**·TFA·H$_2$O (80 mg, 0.10 mmol, 1.0 equiv.) was mixed with anhydrous CHCl$_3$ (2 mL). DIPEA (140 µL, 0.80 µmol, 8 equiv.), BSA (0.40 mL, 1.6 mmol, 16 equiv.) and Me$_3$Si-Cl (38 µL, 0.30 mmol, 3 equiv.) were successively added via a syringe. The resulting suspension was stirred at 50 °C for 1 h until the mixture clarified (conversion >95% by ^{31}P{^{1}H} NMR). Then, t-butyl acrylate (79 µL, 0.50 mmol, 5 equiv.) was added and the solution was stirred at 50 °C overnight. The reaction was quenched with MeOH (0.2 mL) to hydrolyse the silyl esters. After stirring for 5 min, water (~2 mL) was added and the crude product was extracted (3 × 2 mL) into the aqueous phase. Combined aqueous phases were evaporated under vacuum. The oily residue was dissolved in 50% aq. MeOH (2 mL), and the solution was purified by semi-preparative HPLC (C-18, M4). Fractions containing the pure product were combined and the solvents were removed under vacuum. Product **13a** was obtained as a colourless sticky oil (~60 mg, purity ≥95% as determined by NMR; the impurity was assigned to compound **13**), which was directly used in the next step.

Compound **13a**: NMR (D$_2$O, pD ~1.6): ^{1}H δ 1.44 (H$_3$C–C, s, 9H), 1.86–2.03 (CH$_2$–CH$_2$–CH$_2$, m, 2H), 2.06–2.22 (P–CH$_2$–CH$_2$–C(O), m, 2H), 2.23–2.39 (CH$_2$–CH$_2$–CH$_2$, m, 2H), 2.39–2.64 (P–CH$_2$–CH$_2$–C(O) and 2× P–CH$_2$–P, m, 6H), 2.83–3.00 (cycle, m, 2H), 3.00–3.19 (cycle and N–CH$_2$–P, m, 6H), 3.19–3.34 (cycle, m, 2H), 3.34–3.57 (cycle and P–CH$_2$–aryl, m, 12H), 3.57–3.77 (cycle, m, 4H), 7.51–7.62 (o-phenyl, d, $^3J_{HH}$ 8.1, 2H), 8.20–8.30 (m-phenyl, d, $^3J_{HH}$ 8.2, 2H); ^{31}P{^{1}H} δ 24.0–27.0 (bm, 2P), 39.2 (d, $^2J_{PP}$ 9.7, 1P), 45.7 (d, $^2J_{PP}$ 10.0, 1P). ESI-MS: (−) 800.3 (800.3, [M − H]$^−$); (+) 802.3 (802.3, [M + H]$^+$), 824.3 (824.3, [M + Na]$^+$).

The oil from the previous step (TFA salt of **13a**, 60 mg, ~50 µmol) was dissolved in TFA (5 mL) and the solution was stirred at room temperature overnight. The solvents were removed under vacuum and the residue was co-evaporated several times with water to get quantitatively a TFA salt of **13** as a colourless sticky oil (~51 mg, ~50% if calculated as mono-TFA salt; two steps, based on **11**·TFA·H$_2$O).

Compound **13**: NMR (D$_2$O, pD ~1.1): ^{1}H δ 1.89–2.00 (CH$_2$–CH$_2$–CH$_2$, m, 2H), 2.13–2.22 (P–CH$_2$–CH$_2$–CO, dm, $^2J_{HP}$ 15, 2H), 2.27–2.37 (CH$_2$–CH$_2$–CH$_2$, m, 2H), 2.46 (P–CH$_2$–P, pseudo-t, $^2J_{HP}$ 16, 2H), 2.51–2.59 (P–CH$_2$–P, pseudo-t, $^2J_{HP}$ 16, 2H), 2.62 (P–CH$_2$–CH$_2$–C(O), dt, $^3J_{HP}$ 12.6, $^3J_{HH}$ 7.9, 2H), 2.86–3.00 (cycle, m, 2H), 3.04–3.20 (cycle and N–CH$_2$–P, m, 6H), 3.23–3.31 (cycle, m, 2H), 3.34–3.57 (cycle and P–CH$_2$–aryl, m, 12H), 3.57–3.73 (cycle, m, 4H), 7.54–7.58 (o-phenyl, m, 2H), 8.22–8.26 (m-phenyl, dm, $^3J_{HH}$ 8.4, 2H); ^{13}C{^{1}H} δ 20.9 and 21.0 (2× CH$_2$–CH$_2$–CH$_2$, bs), 26.2 (P–CH$_2$–CH$_2$–C(O), d, $^1J_{CP}$ 98), 27.2 (P–CH$_2$–CH$_2$–C(O), s), 32.2–33.5 (2× P–CH$_2$–P, m), 39.2 (P–CH$_2$–aryl, d, $^1J_{CP}$ 89), 47.7–48.6 (cycle, m), 49.9–50.6 (cycle, m), 54.3–55.9 (cycle, m), 57.4–59.4 (cycle, m), 128.6 (m-phenyl, d, J_{CP} 3), 131.6 (o-phenyl, d, J_{CP} 5), 141.4 (p-phenyl, bs), 147.3 (ipso-phenyl, d, J_{CP} 4), 177.1 (C=O, d, $^3J_{CP}$ 16); ^{31}P{^{1}H} δ 23.6–28.3 (m, 2P), 39.5 (d, $^2J_{PP}$ 9.8, 1P), 45.5 (d, $^2J_{PP}$ 8.9, 1P). ESI-MS: (−) 744.2 (744.2, [M − H]$^−$); (+) 746.2 (746.2, [M + H]$^+$). ESI-HR-MS: (−) 744.20940 (744.20898, [C$_{26}$H$_{46}$O$_{12}$N$_5$P$_4$]$^−$), 371.60101 (371.60131, [C$_{26}$H$_{45}$O$_{12}$N$_5$P$_4$]$^{2−}$). HPLC (C-18, M2): R_f ~3.9 min.

Compound 16

In a 100 mL glass flask, **9**·1.5HCl·3.5H$_2$O (110 mg, 120 µmol, 1 equiv.) and 10% Pd/C (22 mg, 20% w/w) were mixed with ~90% aq. AcOH (50 mL). The flask was briefly degassed by using a pump and then gently flushed with hydrogen from a balloon. The reaction mixture was vigorously stirred at 50 °C for 1 d. The solids were filtered off using a syringe microfilter (0.22 µm) and the filtrate was concentrated under reduced pressure. The oily residue was dissolved in 1 : 1 aq. HCl (~5 mL), and the solvents were removed under vacuum. The oil of **16** obtained in this way was immediately used in the next reactions (see below). To obtain a sample for characterization studies, the residue was dissolved in MeOH (~5 mL) and acetone (~50 mL) was added to the solution. The formed suspension was briefly sonicated and the precipitate was filtered off (S4) and washed with acetone (~5 mL) and Et$_2$O (2 × 5 mL). The product was briefly dried in air and under high vacuum to yield the hydrochloride derivative of light-sensitive compound **16**·4HCl·7H$_2$O (98 mg, 80%), which slowly decomposed upon standing, even in the dark, and the compound could not be stored. NMR (D$_2$O, pD ~2): ^{1}H δ 1.79–1.99 (cycle, m, 2H), 1.99–2.19 (P–CH$_2$–P, m, 4H), 2.21–2.42 (cycle, m, 2H), 2.54–3.16 (cycle, m, 8H), 3.16–3.30 (cycle and P–CH$_2$–aryl, m, 6H), 3.30–3.86 (cycle and N–CH$_2$–P, m, 14H), 7.32–7.36 (m-phenyl, d, $^2J_{HH}$ 8, 2H), 7.44–7.48 (o-phenyl, d, $^2J_{HH}$ 8, 2H). ^{13}C{^{1}H} δ 20.6 (cycle, bs), 34.2 (P–CH$_2$–P, pseudo-t, $^1J_{CP}$ 82), 39.3 (P–CH$_2$–C$_{aryl}$, d, $^1J_{CP}$ 90), 49.1 (cycle, s), 49.9 (cycle, bs), 53.9 (cycle, bs), 52.5–57.7 (P–CH$_2$–N and cycle, m), 57.7–59.2 (cycle, bs), 123.6 (m-phenyl, s), 128.8 (p-phenyl, s), 131.9 (o-phenyl, d, $^3J_{CP}$ 5), 136.3 (ipso-phenyl, d, $^2J_{CP}$ 8). ^{31}P{^{1}H} δ 22.8 (N–CH$_2$–P–CH$_2$, m, 2P), 32.6 (CH$_2$–P–Bn, m, 2P). ESI-MS: (−) 747.3 (747.2, [M − H]$^−$); 373.1 (373.1, [M − 2H]$^{2−}$). ESI-HR-MS: (−) 747.27179 (747.27243, [C$_{30}$H$_{50}$O$_8$N$_6$P$_4$]$^−$), 373.13228 (373.13258, [C$_{30}$H$_{50}$O$_8$N$_6$P$_4$]$^{2−}$). Elemental analysis (calc. for C$_{30}$H$_{52}$N$_6$O$_8$P$_4$·4HCl·7H$_2$O, M_R 1020.6): C 35.1 (35.3); H 6.3 (6.9); N 8.4 (8.2); P 12.0 (12.1); Cl 12.6 (13.9). HPLC (C-18, M2): R_f ~3.4 min.

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Org. Biomol. Chem., 2025, **23**, 699–720 | 715

Compound 17

Compound **16** (obtained from **9**·1.5HCl·3.5H$_2$O, 76 mg, 70 μmol) was dissolved in 0.25 M aq. HCl (1.12 mL, 280 μmol, 4.0 equiv.), the solution was cooled in an ice bath (5 °C) and freshly prepared 0.1 M aq. NaNO$_2$ (1.65 mL, 165 μmol, 2.4 equiv.) was added in one portion. The mixture was stirred for 5 min and freshly prepared 0.1 M aq. NaN$_3$ (2.45 mL, 245 μmol, 3.5 equiv.) was added. The vial was removed from the ice bath and the mixture was stirred at room temperature for 3 h (conversion ~95%). The reaction mixture was purified by semi-preparative HPLC (C-8, M1). Fractions with the pure product were pooled, water was added (final volume ~50 mL) and the solution was lyophilized to give **17**·TFA·2H$_2$O as a white foam (38 mg, 57%; over two steps). NMR (D$_2$O, pD ~2): ^{1}H δ 1.89–2.03 (*cycle*, m, 2H), 2.27–2.39 (*cycle* and P–C*H*$_2$–P, m, 6H), 2.60–4.07 (*cycle*, P–C*H*$_2$–aryl and N–C*H*$_2$–P, m, 28H), 7.02–7.06 (*o-phenyl*, d, $^2J_{HH}$ 8, 4H), 7.28–7.32 (*m-phenyl*, d, $^2J_{HH}$ 8, 4H). ^{13}C{^{1}H} δ 20.4 (*cycle*, bs), 32.0–34.0 (P–*C*H$_2$–P, pseudo-t, $^1J_{CP}$ 82), 38.1 (P–*C*H$_2$–aryl, d, $^1J_{CP}$ 91), 48.2 (*cycle*, s), 50.2 (*cycle*, s), 54.4 (*cycle*, s), 55.2 (*cycle*, bs), 56.0–58.2 (P–*C*H$_2$–N and *cycle*, m), 58.2 (*cycle*, s), 119.8 (*m-phenyl*, s), 129.8 (*ipso-phenyl*, d, $^2J_{CP}$ 8 Hz), 131.9 (*o-phenyl*, d, $^3J_{CP}$ 6), 139.0 (*p-phenyl*, s). ^{31}P{^{1}H} δ 22.4 (N–CH$_2$–*P*–CH$_2$, m, 2P); 40.4 (CH$_2$–*P*–Bn, m, 2P). ESI-MS: (−) 799.0 (799.3, [M − H]$^−$); (+) 801.2 (801.3, [M + H]$^+$), 823.2 (823.3, [M + Na]$^+$); (−) 799.3 (799.3, [M − H]$^−$), 399.1 (399.1, [M − 2H]$^{2−}$). ESI-HR-MS: (−) 799.25267 (799.25343, [C$_{30}$H$_{47}$N$_6$O$_8$P$_4$]$^−$), 399.12250 (399.12308, [C$_{30}$H$_{46}$N$_6$O$_8$P$_4$]$^{2−}$). Elemental analysis (calc. for C$_{30}$H$_{48}$N$_{10}$O$_8$P$_4$·TFA·2H$_2$O, M_R 950.7): C 40.3 (40.4); H 5.5 (5.6); N 15.0 (14.7). HPLC (C-8, M1): R_f ~6.5 min. HPLC (C-18, M2): R_f ~4.9 min.

Compound 18

Compound **16** (obtained from **9**·1.5HCl·3.5H$_2$O, 250 mg, 261 μmol) was dissolved in water (30 mL) and CSCl$_2$ (120 μL, 1.57 mmol, ~6 equiv.) dissolved in CCl$_4$ (30 mL) was added. The two-phase mixture was vigorously vortexed at room temperature for 16 h. The mixture was diluted with water (50 mL) and extracted with CH$_2$Cl$_2$ (50 mL). The aqueous layer was separated and evaporated to dryness under vacuum. The residue was purified by semi-preparative HPLC (C-8, M1). Fractions with the pure product were pooled, diluted to 300 mL with water and the solution was lyophilized to give **18**·2.5TFA·1.5H$_2$O as an off-white product (164 mg, 55%, over two steps). NMR (D$_2$O, pD ~2): ^{1}H δ 1.63–2.04 (*cycle*, m, 2H); 2.04–2.54 (*cycle* and P–C*H*$_2$–P, m, 6H); 2.54–4.08 (*cycle*, P–C*H*$_2$–aryl and P–C*H*$_2$–N, m, 28H); 7.06–7.10 (*phenyl*, m, 4H); 7.19–7.23 (*phenyl*, m, 4H). ^{13}C{^{1}H} δ 20.4 (*cycle*, s); 32.1 (P–*C*H$_2$–P, pseudo-t, $^1J_{CP}$ 85); 38.0 (P–*C*H$_2$–aryl, d, $^1J_{CP}$ 91); 47.6 (*cycle*, s); 49.5 (*cycle*, s); 52.8–55.6 (P–*C*H$_2$–N and *cycle*, m); 56.4–59.5 (*cycle*, m); 125.9 (*phenyl*, s); 129.3 (*phenyl*, s); 131.2 (*phenyl*, s); 132.0 (*phenyl*, s); 134.8 (N*C*S, s). ^{31}P{^{1}H} δ 24.9 (N–CH$_2$–*P*, bs), 39.3 (*P*–CH$_2$–aryl, s). ESI-MS: (−) 831.2 (831.2, [M − H]$^−$); (+) 833.2 (833.2, [M + H]$^+$). ESI-HR-MS: (+) 833.19947 (833.19983, [C$_{32}$H$_{49}$N$_6$O$_8$P$_4$S$_2$]$^+$); (−) 831.18577 (831.18527, [C$_{32}$H$_{47}$N$_6$O$_8$P$_4$S$_2$]$^−$). Elemental analysis (calc. for C$_{32}$H$_{48}$N$_6$O$_8$P$_4$S$_2$·2.5TFA·1.5H$_2$O, M_R 1144.9): C 38.7 (38.8); H 4.3 (4.7); N 7.1 (7.3). HPLC (C-18, M2): R_f ~5.4 min.

Compound 19

In a 100 mL flask, the pyridinium salt of **10** (**10**·py·3.5H$_2$O, 1.4 g, 1.8 mmol) was dissolved in ~75% aq. AcOH (50 mL), Pd/C (0.14 g, 10% w/w) was added and the flask was well-flushed with hydrogen gas. A hydrogen balloon was attached and the suspension was vigorously stirred at 50 °C for 2 d. The reaction mixture was cooled to room temperature, the solids were filtered off through a syringe microfilter (0.22 μm) and the solvents were removed under vacuum. The oily residue was successively co-evaporated with toluene (10 mL), conc. aq. HCl (10 mL), water (10 mL) and MeOH (10 mL). The oily residue was dissolved in MeOH (5 mL) and acetone (50 mL) was slowly added leading to the precipitation of a solid. The solid was filtered off, washed with Et$_2$O (3 × 10 mL) and immediately dissolved in water (5 mL). The solution containing the crude product was purified by flash chromatography (C-18, M2). Fractions containing the pure product were combined and the solvents were evaporated under vacuum. The oily residue was dissolved in water (200 mL) and the solution was lyophilized to give zwitterionic **19**·4H$_2$O as a fluffy solid (0.95 g, 72%). NMR (D$_2$O, pD ~2): ^{1}H δ 1.90–2.08 (*cycle*, m, 2H), 2.15–2.29 (P–C*H*$_2$–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 16.2, 2H), 2.19–2.35 (*cycle*, m, 2H), 2.25–2.37 (P–C*H*$_2$–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 18, 2H), 2.70–4.19 (*cycle*, N–C*H*$_2$–P and P–C*H*$_2$–aryl, m, 26H); 7.37–7.41 (*o-phenyl*, d, 2H, $^3J_{HH}$ 8), 7.48–7.52 (*m-phenyl*, d, 2H, $^3J_{HH}$ 8); ^{13}C{^{1}H} δ 20.2 (*cycle*, s), 20.5 (*cycle*, s), 32.9 (P–*C*H$_2$–P, dd, $^1J_{CP}$ 118, $^1J_{CP}$ 82), 33.0–34.2 (P–*C*H$_2$–P, pseudo-t, $^1J_{CP}$ 83), 38.8 (P–*C*H$_2$–aryl, d, $^1J_{CP}$ 91), 47.3–58.7 (*cycle*, m), 123.7 (*m-phenyl*, d, $^4J_{CP}$ 3), 128.8 (*p-phenyl*, s), 131.9 (*o-phenyl*, d, $^3J_{CP}$ 5), 135.6 (*ipso-phenyl*, d, $^2J_{CP}$ 7); ^{31}P{^{1}H} δ 15.0–15.6 (*P*O$_3$H$_2$, m, 1P), 22.2–22.8 (*P*–CH$_2$–N, m, 1P), 23.1–23.7 (*P*–CH$_2$–N, m, 1P), 35.1–35.7 (*P*–CH$_2$–aryl, m, 1P). ESI-MS: (−) 658.2 (658.2, [M − H]$^−$), (+) 660.2 (660.2, [M + H]$^+$), 682.2 (682.2 [M + Na]$^+$). ESI-HR-MS: (−) 658.20912 (658.20950, [C$_{23}$H$_{44}$O$_9$N$_5$P$_4$]$^−$); (+) 680.19127 (680.19144, [C$_{23}$H$_{44}$O$_9$N$_5$P$_4$Na]$^+$). TLC (i-PrOH–conc. aq. NH$_3$–H$_2$O 7:3:3): R_f ~0.1. Elemental analysis (calc. for C$_{23}$H$_{45}$N$_5$O$_9$P$_4$·4H$_2$O, M_R 731.6): C 37.6 (37.8), H 7.1 (7.3), N 9.6 (9.6), P 16.1 (16.9). HPLC (C-18, M2): R_f ~1.8 min.

Compound 20

Compound **19**·4H$_2$O (250 mg, 0.34 mmol, 1 equiv.) was dissolved in 1% aq. HCl (20 mL) and the solution was cooled in an ice bath. An aqueous solution (2 mL) of NaNO$_2$ (35 mg, 0.51 mmol, 1.5 equiv.) was added and, after a few minutes, an aqueous solution (3 mL) of NaN$_3$ (45 mg, 0.69 mmol, 2 equiv.) was added. The reaction mixture was left to warm up to room temperature over 3 h. The volatiles were evaporated under vacuum. The oily residue was dissolved in water (2 mL) and the crude product was purified by flash chromatography (C-18, M2). Fractions containing the pure product were combined and the solvents were evaporated to dryness under vacuum. The oily residue was dissolved in water (50 mL) and lyophilized. The product was obtained as **20**·2HCl·4H$_2$O (fluffy solid,

716 | *Org. Biomol. Chem.*, 2025, **23**, 699–720

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180 mg, 63%). NMR (D$_2$O, pD ~3): ^{1}H δ 1.92–2.08 (*cycle*, m, 2H), 2.23–2.37 (P–C*H$_2$*–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 15.9, 2H), 2.27–2.39 (*cycle*, m, 2H), 2.30–2.42 (P–C*H$_2$*–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 19, 2H), 2.71–4.12 (*cycle*, N–C*H$_2$*–P and P–C*H$_2$*–aryl, m, 26H), 7.10–7.14 (*o-phenyl*, d, 2H, $^3J_{HH}$ 8), 7.36–7.40 (*m-phenyl*, d, 2H, $^3J_{HH}$ 8); ^{13}C{^{1}H} δ 20.4 (*cycle*, s), 20.6 (*cycle*, s), 31.9–34.1 (P–*C*H$_2$–P, m), 38.4 (P–*C*H$_2$–aryl, d, $^1J_{CP}$ 92), 46.9–60.1 (*cycle*, m), 119.8 (*m-phenyl*, s), 130.3 (*p-phenyl*, s), 132.0 (*o-phenyl*, d, $^3J_{CP}$ 5), 139.0 (*ipso-phenyl*, d, $^2J_{CP}$ 4); ^{31}P{^{1}H} δ 15.7–16.3 (*P*O$_3$H$_2$, m, 1P), 21.4–23.4 (*P*–CH$_2$–N, m, 1P), 22.1–24.7 (*P*–CH$_2$–N, m, 1P), 38.2–39.8 (*P*–CH$_2$–C, m, 1P). ESI-MS: (−) 684.2 (684.2, [M − H]$^−$), 341.6 (341.6, [M − 2H]$^{2−}$); (+) 686.2 (686.2, [M + H]$^+$), 708.2 (708.2, [M + Na]$^+$). ESI-HR-MS: (+) 684.19969 (684.20000, [C$_{23}$H$_{42}$O$_9$N$_7$P$_4$]$^+$), 341.59624 (341.59636, [C$_{23}$H$_{41}$O$_9$N$_7$P$_4$]$^{2+}$). TLC (*iPrOH–conc. aq. NH$_4$OH–H$_2$O 7 : 3 : 3*): R_f ~0.2. Elemental analysis (calc. for C$_{23}$H$_{43}$N$_7$O$_9$P$_4$·2HCl·4H$_2$O, M_R 830.5): C 33.2 (33.3); H 6.0 (6.4); N 11.4 (11.8), P 14.8 (14.9), Cl 9.1 (8.5). HPLC (C-8, M1): R_f ~5.7 min.

Compound 21

Compound **19**·4H$_2$O (150 mg, 0.205 mmol, 1 equiv.) was dissolved in 1% aq. HCl (4 mL) and a solution of thiophosgene (47 µL, 0.61 mmol, 3 equiv.) in CCl$_4$ (4 mL) was added. The mixture was vigorously stirred at room temperature for 1 d. The aqueous phase was separated and evaporated under vacuum. The oily residue was dissolved in water (2 mL) and the crude product was purified by flash chromatography (C-18, M2). Fractions containing the pure product were combined and the solvents were evaporated to dryness under vacuum. The oily residue was dissolved in water (50 mL) and lyophilized. Product **21**·2HCl·4H$_2$O was isolated as a fluffy solid (105 mg, 60%). NMR (D$_2$O, pD ~2): ^{1}H δ 1.94–2.10 (CH$_2$–C*H$_2$*–CH$_2$, m, 2H), 2.22–2.36 (P–C*H$_2$*–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 16, 2H), 2.26–2.38 (CH$_2$–C*H$_2$*–CH$_2$, m, 2H), 2.31–2.43 (P–C*H$_2$*–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 19, 2H), 2.86–3.92 (*cycle*, N–C*H$_2$*–P and P–C*H$_2$*–C, m, 26H), 7.35–7.39 (*o-phenyl*, d, 2H, $^3J_{HH}$ 8 Hz), 7.37–7.41 (*m-phenyl*, m, 2H); ^{13}C{^{1}H} δ 20.8 (CH$_2$–*C*H$_2$–CH$_2$, s), 21.3 (CH$_2$–*C*H$_2$–CH$_2$, s), 31.7 (P–*C*H$_2$–P, dd, $^1J_{CP}$ 120, $^1J_{CP}$ 83), 31.7–33.0 (P–*C*H$_2$–P, m), 38.4 (P–*C*H$_2$–aryl, d, $^1J_{CP}$ 91), 46.6–59.2 (*cycle*, m), 126.7 (*m-phenyl*, s), 130.1 (*p-phenyl*, s), 131.8 (*o-phenyl*, s), 132.2 (*ipso-phenyl*, d, $^2J_{CP}$ 8 Hz), 134.8 (*C*=S, s); ^{31}P{^{1}H} δ 16.0–16.6 (*P*O$_3$H$_2$, m, 1P), 22.4–24.4 (*P*–CH$_2$–N, m, 1P), 22.4–24.8 (*P*–CH$_2$–N, m, 1P), 37.7–39.1 (*P*–CH$_2$–aryl, m, 1P). ESI-MS: (−) 700.2 (700.2, [M − H]$^−$), 349.6 (349.6, [M − 2H]$^{2−}$); (+) 702.2 (702.2, [M + H]$^+$). ESI-HR-MS: (−) 700.16563 (700.16592, [C$_{24}$H$_{42}$O$_9$N$_5$P$_4$S]$^−$), 349.57921 (349.57932, [C$_{24}$H$_{41}$O$_9$N$_5$P$_4$S]$^{2−}$). Elemental analysis (calc. for C$_{24}$H$_{43}$N$_5$O$_9$P$_4$S·2HCl·4H$_2$O, M_w 846.6): C 34.7 (34.1), H 6.1 (6.3), N 8.1 (8.3), P 14.6 (14.6), S 3.3 (3.8), Cl 8.8 (8.4). HPLC (C-8, M3): R_f ~16 min.

Compound 22

The trifluoroacetate salt of **13** obtained above (~51 mg) was dissolved in ~75% aq. AcOH (10 mL) and Pd/C (9 mg, 20% w/w) was added. The flask was briefly degassed and then flushed with hydrogen gas. A hydrogen balloon was attached and the suspension was vigorously stirred at 50 °C for 4 h. The reaction mixture was cooled to room temperature, the solids were filtered off through a syringe microfilter (0.22 µm) and the solvents were removed under vacuum. The oily residue was successively co-evaporated with toluene (5 mL), conc. aq. HCl (5 mL) and water (5 mL). Compound **22** was isolated as an oil (~30 mg), which was used directly in the next step. NMR (D$_2$O, pD <1): ^{1}H δ 1.88–2.04 (CH$_2$–C*H$_2$*–CH$_2$, m, 2H), 2.15–2.40 (CH$_2$–C*H$_2$*–CH$_2$ and P–C*H$_2$*–CH$_2$, m, 4H), 2.44–2.53 (P–C*H$_2$*–P, m, 2H), 2.53–2.72 (CH$_2$–C*H$_2$*–CO and P–C*H$_2$*–P, m, 4H), 2.87–3.04 (*cycle*, m, 2H), 3.06–3.23 (*cycle* and N–C*H$_2$*–P, m, 6H), 3.24–3.33 (*cycle*, m, 2H), 3.45 (P–C*H$_2$*–aryl, d, $^2J_{HP}$ 17.6, 2H), 3.33–3.74 (*cycle*, m, 14H), 7.40–7.45 (*o-phenyl*, d, 2H, $^3J_{HH}$ 7.8 Hz), 7.46–7.52 (*m-phenyl*, m, 2H); ^{13}C{^{1}H} δ 21.0 (2× CH$_2$–*C*H$_2$–CH$_2$, bs), 26.2 (P–CH$_2$–*C*H$_2$–CO, d, $^1J_{CP}$ 101), 27.2 (P–CH$_2$–*C*H$_2$–CO, bs), 2× 31.4–33.7 (2× P–*C*H$_2$–P, m), 38.3 (P–*C*H$_2$–aryl, d, $^1J_{CP}$ 91), 47.6–48.4 (*cycle*, m), 49.9–50.8 (*cycle*, m), 54.3–56.0 (*cycle*, m), 57.2–59.2 (*cycle*, m), 124.1 (*m-phenyl*, bs), 129.4 (*o-phenyl*, bs), 132.2 (*o-phenyl*, bs), 134.0 (*i-phenyl*, bs), 177.1 (*C*=O, d, $^3J_{CP}$ 15); ^{31}P{^{1}H} δ 23.8–28.2 (m, 2P), 41.3–42.1 (m, 1P), 45.4–46.2 (m, 1P). ESI-MS: (−) 714.2 (714.2, [M − H]$^−$), 356.6 (356.6, [M − 2H]$^{2−}$); (+) 716.2 (716.2, [M + H]$^+$). ESI-HR-MS: (−) 714.23547 (714.23571, [C$_{26}$H$_{48}$O$_{10}$N$_5$P$_4$]$^−$), 356.61398 (356.61422, [C$_{26}$H$_{47}$O$_{10}$N$_5$P$_4$]$^{2−}$). HPLC (C-18, M2): R_f ~3.5 min.

Compound 23

The oily hydrochloride salt of **22** obtained above (~30 mg) was dissolved in 1% aq. HCl (5 mL) and the solution was cooled in an ice bath. An aqueous solution (1 mL) of NaNO$_2$ (4.0 mg, 58 µmol, 1.5 equiv.) was added and, after a few minutes, an aqueous solution (1 mL) of NaN$_3$ (5.0 mg, 77 µmol, 2 equiv.) was added. The solution was left to warm up to room temperature over 3 h. The solution was concentrated under vacuum to ~2 mL and the crude product was purified by semi-preparative HPLC (C-18, M5). Fractions containing the pure product were combined and directly lyophilized. The product was isolated as **23**·1.5TFA·H$_2$O (fluffy solid, 13 mg, yield ~18%, over four steps, based on **11**·TFA·H$_2$O, 80 mg). NMR (D$_2$O, pD ~1.6): ^{1}H δ 1.86–2.01 (CH$_2$–C*H$_2$*–CH$_2$, m, 2H), 2.15 (P–C*H$_2$*–CH$_2$, dt, $^2J_{HP}$ 14.9, $^3J_{HH}$ 7.5, 2H), 2.26–2.38 (CH$_2$–C*H$_2$*–CH$_2$, m, 2H), 2.41 (P–C*H$_2$*–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 16, 2H), 2.47 (P–C*H$_2$*–P, dd, $^2J_{PH}$ 16.1, $^2J_{PH}$ 12.9, 2H), 2.62 (CH$_2$–C*H$_2$*–CO, dt, $^3J_{HP}$ 12.2, $^3J_{HH}$ 8.0, 2H), 2.81–2.99 (*cycle*, m, 2H), 2.97–3.20 (*cycle* and N–C*H$_2$*–P, m, 6H), 3.22–3.32 (*cycle*, m, 2H), 3.35 (P–C*H$_2$*–aryl, d, $^2J_{HP}$ 17.1, 2H), 3.40–3.59 (*cycle*, m, 10H), 3.60–3.72 (*cycle*, m, 4H), 7.08–7.14 (*o-phenyl*, dm, 2H, $^3J_{HH}$ 8.2 Hz), 7.33–7.40 (*m-phenyl*, m, 2H); ^{13}C{^{1}H} δ 20.7 (2× CH$_2$–*C*H$_2$–CH$_2$, bs), 26.3 (P–*C*H$_2$–CH$_2$–CO, d, $^1J_{CP}$ 98), 27.4 (P–CH$_2$–*C*H$_2$–CO, bs), 31.9–33.9 (2× P–*C*H$_2$–P, m), 38.2 (P–*C*H$_2$–aryl, d, $^1J_{CP}$ 91), 47.8–48.7 (*cycle*, m), 49.8–50.7 (*cycle*, m), 54.2–55.7 (*cycle*, m), 57.3–59.0 (*cycle*, m), 120.0 (*m-phenyl*, d, $^4J_{CP}$ 3), 129.5 (*p-phenyl*, s), 132.1 (*o-phenyl*, d, $^3J_{CP}$ 5), 139.3 (*ipso-phenyl*, d, $^2J_{CP}$ 4), 177.3 (*C*=O, d, $^3J_{CP}$ 16); ^{31}P{^{1}H} δ 21.9–25.8 (bm, 2P), 41.0–42.0 (m, 1P), 43.5–44.7 (m, 1P). ESI-MS: (−) 740.2 (740.2, [M − H]$^−$), 369.6

(369.2, $[M - 2H]^{2-}$); (+) 742.2 (742.2, $[M + H]^+$), 764.2 (764.2, $[M + Na]^+$). ESI-HR-MS: (−) 740.22574 (740.22621, $[C_{26}H_{46}O_{10}N_7P_4]^-$), 369.60920 (369.60947, $[C_{26}H_{45}O_{10}N_7P_4]^{2-}$). Elemental analysis (calc. for $C_{26}H_{47}N_7O_{10}P_4\cdot1.5TFA\cdot H_2O$, M_w 930.6): C 37.8 (37.4), H 5.4 (5.5), N 10.1 (10.5). HPLC (C-18, M2): R_f ~4.1 min.

Compound 25

In a 4 mL glass vial, **19**·H$_2$O (33 mg, 45.5 μmol) and the NHS-ester of 3-[4-(carboxymethyl)-phenyl]-6-methyl-1,2,4,5-tetrazine (16.4 mg, 50.1 μmol, 1.1 equiv.) were dissolved in a mixture of MES–NaOH aq. buffer (1.0 M, pH 6.2, 1.35 mL, 1.35 mmol, 25 equiv.) and MeCN (2.0 mL). Water (870 μL) was added, and the reaction mixture was stirred at room temperature for 2 d. The volatiles were evaporated, and the reaction mixture was purified by semi-preparative HPLC (C-8, M1). Fractions containing the pure product were transferred to a 100 mL round-bottomed flask with water (~20 mL) and the solution (~60 mL) was lyophilized. The product (≥99% purity as determined by NMR) was obtained as a fine pink powder of **25**·0.5TFA·1.5H$_2$O (19 mg, 42%). NMR (D$_2$O, pD ~2): ^{1}H δ 1.92–2.02 (CH$_2$–CH$_2$–CH$_2$, m, 2H), 2.21–2.31 (CH$_2$–CH$_2$–CH$_2$, m, 2H), 2.25–2.37 (P–CH$_2$–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 16, 2H), 2.30–2.42 (P–CH$_2$–P, pseudo-t, $^2J_{HP}$–$^2J_{HP}$ 19, 2H), 2.71–3.86 (cycle, N–CH$_2$–P, P–CH$_2$–aryl and tetrazine-CH$_3$, m, 29H), 3.93 (CH$_2$–CO, s, 2H), 7.36–7.40 (phenyl, dm, 2H, $^3J_{HH}$ 8), 7.44–7.48 (phenyl, dm, 2H, $^3J_{HH}$ 8), 7.65–7.69 (phenyl, dm, 2H, $^3J_{HH}$ 8), 8.41–8.45 (phenyl, dm, 2H, $^3J_{HH}$ 8); ^{13}C{^{1}H} δ 20.4 (CH$_2$–CH$_2$–CH$_2$, s), 20.6 (CH$_2$–CH$_2$–CH$_2$, s), 20.7 (CH$_3$, s), 31.6–34.0 (2× P–CH$_2$–P, m), 38.6 (P–CH$_2$–aryl, d, $^1J_{CP}$ 91), 43.6 (CH$_2$–CO, s), 47.4–58.7 (cycle, m), 126.7 (m-phenyl, s), 130.1 (p-phenyl, s), 131.8 (o-phenyl, s), 132.2 (ipso-phenyl, d, $^2J_{CP}$ 8), 164.7 (N$_2$–C–N$_2$, s), 167.9 (N$_2$–C–N$_2$, s), 173.2 (CO, s). ^{31}P{^{1}H} δ 15.7–16.3 (PO$_3$H$_2$, m, 1P), 21.0–23.0 (P–CH$_2$–N, m, 1P), 22.0–25.0 (P–CH$_2$–N, m, 1P), 38.0–39.6 (P–CH$_2$–aryl, m, 1P). ESI-MS: (−) 870.3 (870.3, $[M - H]^-$); (+) 872.5 (872.3, $[M + H]^+$), 894.5 (894.3, $[M + Na]^+$). Elemental analysis (calc. for $C_{34}H_{53}N_9O_{10}P_4\cdot0.5TFA\cdot1.5H_2O$, M_R 955.8): C 43.9 (44.0), H 5.5 (6.0), N 12.8 (13.2). HPLC (C-8, M1): R_f ~6.2 min.

Compound 26

In a 4 mL glass vial, **18**·2.5TFA·1.5H$_2$O (45 mg, 39 μmol, 1 equiv.) and N-(2-aminoethyl)maleimide hydrochloride (7.0 mg, 39 μmol, 1 equiv.) were dissolved anhydrous DMF (~2 mL) and anhydrous iPr$_2$NEt (35 μL, 200 μmol, 5 equiv.) was added. The mixture was vigorously stirred at room temperature for 5 h, during which time the solution turned into a cloudy suspension. Then, 0.1% aq. TFA (~2 mL) was added and the mixture was stirred until the solution became clear. The aqueous solution was washed with AcOEt (2 × 2 mL) and then directly purified by semi-preparative HPLC (C-18, M7). Fractions containing the pure products were combined and directly lyophilized. The products **26a**·2TFA·3H$_2$O (13 mg, 24%; obtained as a mixture of two conformers) and **26**·2TFA·2H$_2$O (21 mg, 43%) were isolated as fluffy solids. Some unreacted **18**·2.5TFA·1.5H$_2$O (9 mg, 20%) was regenerated after purification.

Compound 26: NMR (D$_2$O, pD ~1.8): ^{1}H δ 1.65–2.05 (CH$_2$–CH$_2$–CH$_2$, m, 2H), 2.11–2.56 (CH$_2$–CH$_2$–CH$_2$ and P–CH$_2$–P, m, 6H), 2.64–3.15 (cycle, m, 8H), 3.15–3.33 (cycle, m, 8H), 3.33–3.91 (cycle, N–CH$_2$–P and C(S)NH–CH$_2$–CH$_2$–NC(O), m, 16H), 6.65–6.91 (CH–CO, bs, 4H), 7.09–7.22 (phenyl, m, 4H), 7.22–7.38 (phenyl, m, 4H). ^{13}C{^{1}H} δ 20.0–21.9 (2× CH$_2$–CH$_2$–CH$_2$, bs), 31.7–33.9 (2× P–CH$_2$–P, m), 37.9 (CH$_2$–NHCS, s), 38.0–39.1 (2× P–CH$_2$–aryl, m), 43.6 (CH$_2$–NCO, s), 47.8–48.8 (cycle, m), 49.6–51.0 (cycle, m), 54.2–55.8 (cycle and P–CH$_2$–N, m), 58.2–59.5 (cycle, m), 126.4–127.0 (phenyl, m), 129.4–130.0 (phenyl, m), 131.5–132.3 (phenyl, m), 132.7–133.2 (phenyl, m), 135.0–135.5 (CH–CO, s), 173.4 (NCO, s), 180.7 (NHCS, s). ^{31}P{^{1}H} δ 21.2–27.6 (P–CH$_2$–N, m, 2P), 37.6–40.1 (P–CH$_2$–aryl, m, 1P), 40.1–41.9 (P–CH$_2$–aryl, m, 1P). ESI-MS: (−) 971.2 (971.2, $[M - H]^-$), 485.1 (485.1, $[M - 2H]^{2-}$). ESI-HR-MS: (−) 971.24303 (971.24385, $[C_{38}H_{55}N_8O_{10}P_4S_2]^-$). Elemental analysis (calc. for $C_{38}H_{56}N_8O_{10}P_4S_2\cdot2TFA\cdot2H_2O$, M_R 1237.0): C 40.6 (40.8); H 4.7 (5.0); N 8.9 (9.1); P 9.9 (10.0); S 5.2 (5.2). HPLC (C-18, M2): R_f ~4.9 min.

Compound 26a (a major conformer): NMR (D$_2$O, pD ~1.8): ^{1}H δ 1.79–2.06 (CH$_2$–CH$_2$–CH$_2$, m, 2H), 2.18–2.36 (CH$_2$–CH$_2$–CH$_2$, m, 2H), 2.36–2.50 (P–CH$_2$–P, m, 4H), 2.76–3.19 (cycle, m, 8H), 3.19–3.36 (cycle, m, 8H), 3.36–3.60 (cycle and N–CH$_2$–P, m, 12H), 3.60–3.86 (C(S)NH–CH$_2$–CH$_2$–NC(O), m, 8H), 6.85 (CH–C(O), s, 4H), 7.10–7.24 (phenyl, m, 4H), 7.29–7.40 (phenyl, m, 4H). ^{13}C{^{1}H} δ 20.0–21.4 (CH$_2$–CH$_2$–CH$_2$, bs), 32.1–33.5 (P–CH$_2$–P, m), 37.9 (CH$_2$–NHC(S), s), 38.4 (P–CH$_2$–aryl, d, $^1J_{CP}$ 90), 43.5 (CH$_2$–NC(O), s), 44.2–45.3 (cycle, m), 47.7–48.7 (cycle, m), 49.8–50.8 (cycle, m), 54.2–55.8 (cycle and P–CH$_2$–N, m), 58.0–59.6 (cycle, m), 126.5–126.8 (phenyl, m), 131.6–132.1 (phenyl, m), 132.0–132.4 (phenyl, m), 135.3 (CH–C(O), s), 135.5–136.0 (phenyl, m), 173.7 (NC(O), s), 180.5 (NHC(S)NH s). ^{31}P{^{1}H} δ 17.5–27.5 (P–CH$_2$–N, m, 2P), 36.8–43.5 (P–CH$_2$–aryl, m, 2P). ESI-MS: (−) 1111.3 (1111.3, $[M - H]^-$), 555.1 (555.1, $[M - 2H]^{2-}$). ESI-HR-MS: (−) 1111.30173 (1111.30243, $[C_{44}H_{61}N_{10}O_{12}P_4S_2]^-$), 555.14685 (555.14758, $[C_{44}H_{60}N_{10}O_{12}P_4S_2]^{2-}$). Elemental analysis (calc. for $C_{44}H_{64}N_{10}O_{12}P_4S_2\cdot2TFA\cdot3H_2O$, M_R 1395.2): C 41.1 (41.3); H 5.0 (5.2); N 10.1 (10.0). HPLC (C-18, M2): R_f ~4.5 min.

Radiolabelling experiments

For ligand labelling, stock solutions (1 mM) of the ligands were prepared from the solid samples by dissolving them in water. These solutions were carefully diluted to obtain stock solutions (10–600 μM) for the labelling experiments. For ligand comparative studies, aq. solutions (1 mM) of the ligands were prepared from the solids and they were diluted to obtain stock solutions (100 μM) for the labelling experiments. A solution of a ligand in MES buffer (prepared by mixing 1 μL of the ligand stock solution with $c_L = 100$ μM and 10 μL of the MES stock solution with pH 6.2/5.6 and $c = 0.5$ M) was pre-heated to 25 °C. To this solution, freshly prepared (within less than 1 h) [^{64}Cu]CuCl$_2$ in aq. HCl (6 μL, 9–11 MBq) was added to get the final samples (17 μL, Cu : L molar ratio approx. 1 : 85–1 : 95). The samples were incubated for 10 min (pH 6.2) or 1 h (pH 5.6) at 25 °C. For all ligands, the experiments were always done with three different batches of [^{64}Cu]CuCl$_2$. The

718 | Org. Biomol. Chem., 2025, **23**, 699–720

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labelling progress was checked by TLC analysis (0.5 μL of the mixture, together with 0.5 μL [^{64}Cu]CuCl$_2$ stock solution) performed in aq. EDTA (0.1 M, pH ~5): R_f 0.8–0.9 (free Cu), start (complexes of the phosphorus ligands), 0.1–0.2 (carboxylate complexes).

SOD stability test

To ensure quantitative incorporation of ^{64}Cu into the in-cage complex with all ligands in this experiment, ~6000 equiv. of the chelators with respect to the molar amount of [^{64}Cu]CuCl$_2$ was used as well as a long radiolabelling time (~2 h incubation at room temperature was used for all complexes, except that of H$_2$cb-te2a where heating to 50 °C had to be applied). The SOD stability test was carried according to the literature procedure.[47] Briefly, human erythrocyte superoxide dismutase (SOD) was reconstituted in water to a protein concentration of 1 μg μL^{-1} (~4 units per μL) and stored in aliquots (10 μL, 0.3 nmol) at −20 °C. For the SOD experiments, aliquots were thawed on ice and [^{64}Cu]Cu-labelled ligands 1–4 and the "standard" ligands (0.1 nmol, 1.5 μL) or [^{64}Cu]CuCl$_2$ as a reference were added to SOD (0.3 nmol, 10 μg). The mixtures were incubated at 37 °C for 1 h followed by adding one volume of native sample buffer (Bio-Rad Laboratories, cat. #161-0738). The samples were separated using non-reducing and non-denaturing polyacrylamide gel electrophoresis (PAGE) with acrylamide concentrations of 15% in the resolving gel and 5% in the stacking gel; 20 μL of each sample were loaded into each well of the gel. The native PAGE was run at room temperature and 80 V until the dye front reached the resolving gel and then increased up to 140–160 V. After electrophoresis, the gel was washed with water for 1 min and exposed to a reusable imaging plate (Fujifilm) for 10 min. Following electronic autoradiography using a radioluminography laser scanner, the gel was stained with PageBlue protein staining solution (Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative analysis of average band intensities was performed with the Advanced Image Data Analysis (AIDA) program (Raytest).

Author contributions

TD, VK and PH conceptualized the project and VK, HJP and PH supervised different parts of the realization. PU, TD and VH conducted syntheses and characterization. TD and HJP conducted the radiochemical experiments. PU, TD and VK wrote the original draft, and all co-authors contributed to the reviewing and editing process.

Data availability

Crystallographic data for compound **C** (pno2ppinb) has been deposited at the CCDC (https://www.ccdc.cam.ac.uk/) under no. 2341020.†

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

Conflicts of interest

The authors declare the following competing financial interest (s): T. David, V. Kubíček and P. Hermann have filed a patent application containing some data presented in the manuscript.

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