ORIGINAL ARTICLE

Synthesis and evaluation of resins bearing substrate-like inhibitor functions for capturing copper amine oxidases

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The aim of this work was to make new tools available for investigating the interactions between enzymes and substrate-like inhibitors in copper amine oxidases (CAOs), a class of enzymes that controls important cellular processes, such as the crosslinking of elastin and collagen, cell proliferation and the regulation of intracellular polyamines. Starting from previous work, we synthesized the poly(*N,N*-dimethylacrylamide)-based resin R2 and the new TentaGel resins T3 and T4 obtained by ether bonding CAO substrate-like inhibitor moieties onto commercial TentaGel S-Br, which contains bromomethyl groups susceptible to nucleophilic substitution reactions. We used polyacrylamide gel electrophoresis (PAGE) experiments to determine the capability of the prepared resins to capture plasma amine oxidase (PAO) and diamine oxidase (DAO), members of the CAO family. The poly(*N,N*-dimethylacrylamide)-based resin R2 was able to block PAO and DAO, being the first insoluble polymeric material capable of capturing enzymes of the CAO family.

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INTRODUCTION

Copper amine oxidases (CAOs, EC 1.4.3.6) are ubiquitous enzymes found in prokaryotes and eukaryotes, humans included, where they control important cellular processes, such as the crosslinking of elastin and collagen, cell proliferation and the regulation of intracellular polyamines. With the exception of lysyl oxidase (32 kDa), CAOs are homodimers with subunit molecular weights between 70 kDa and 95 kDa, and with one copper ion and a quinone cofactor per subunit. CAOs are also characterized by the presence of cysteine residues and a variable percentage of carbohydrates, depending on the enzyme source.

The enzymatic reaction results in the deamination of aminomethyl substrates to produce aldehydes, ammonia and reduced molecular oxygen in the form of hydrogen peroxide.

Knowledge concerning the physiological role, mechanism of action^{1–4} and numerous X-ray structures^{5–14} of these enzymes, as well as methods for protein expression of CAOs,¹⁵ making larger quantities of protein samples available, which is useful for various experiments, has grown progressively in the past two decades.

Remarkably, one of the X-ray structures indicates that the complex between 2-hydrazinopyridine, a non-substrate-like, nonselective, carbonyl group scavenger inhibitor, and *Escherichia coli* amine oxidase results in the formation of an imine double bond between the NH₂ of the inhibitor and the C-5 carbonyl group of the cofactor 2,4,5-trihydroxyphenylalanine quinone.¹⁶

Our contribution in this field mainly focuses on the synthesis of the first substrate-like inhibitors classified as highly selective, partially reversible for benzylamine oxidase (BAO) having a benzylamine structure,¹⁷ highly selective, fully reversible for BAO having a 4-aminomethylpyridine structure,¹⁸ and highly active, nonselective for BAO and diamine oxidase (DAO) having a 4-aminomethylpyridine structure.¹⁸

As CAOs are characterized by a moderate substrate selectivity, making their biological role rather complex and difficult to elucidate, further research is needed. Purposefully developed chemical agents, either as small molecules or polymeric soluble and insoluble structures, that are active as substrates or as substrate-like inhibitors that are highly selective or nonselective would also be of use. By performing enzymatic reactions or promoting the formation of enzyme–inhibitor complexes or capturing the enzyme, these agents could help to clarify the enzyme–substrate interactions responsible for the specific or complementary natural role of each member of the CAO family, thus contributing to a deeper knowledge of possible correlated pathologies and treatments.

Selective and nonselective, completely or partially reversible substrate-like inhibitors could allow the formation of complexes with CAOs that would most likely be more significant than those obtainable with non-substrate-like, nonselective, carbonyl group scavenger inhibitors. Moreover, insoluble polymers or resins endowed with reversible inhibitory activity could also prove useful as tools for enzyme separation and purification.

Among CAOs, only lysyl oxidase is naturally devoted to oxidize lysine residues in macromolecular structures such as elastin and collagen, while all the other enzymes of the same class oxidize small molecules. Nevertheless, in a previous study,¹⁹ we prepared the first soluble copolymers (**P1a** and **P1c**, Scheme 1) and resin (**R1a**, Scheme 1) containing aminoalkylbenzene residues, which were very good substrates of lysyl oxidase and BAO, with **P1c** also being active toward DAO. Interestingly, we found that **R1a** was as active toward lysyl oxidase as the natural substrate elastin, and **P1c** was 9.7 times more active than elastin itself.

In that study, we also prepared resin **R2** (Scheme 1) with expected inhibitory activity, containing benzylamine residues and bearing two different alkoxy groups at positions 2 and 6. One of these alkoxy groups contained a polymerizable function. However, its biological behavior toward CAOs, in spite of its potentially high interest, could not be studied due to our delay in setting up convenient bio-tests, now attained, for the CAO proteins interacting with fully insoluble polymeric materials. We designed resin **R2** by placing the polymerizable 4-vinylbenzyloxy group at one of the two positions *ortho* to the aminomethyl group in a trisubstituted benzene ring, deferring to future research the exploration of 2,6-dialkoxybenzylamine residues having proper alkoxy groups at positions 2 and 6, and placing a polymerizable function at a different position in a tetrasubstituted benzene ring.

In this work, we expanded upon previous research and attained useful results. We re-prepared the highly hydrophilic insoluble *N,N*-dimethylacrylamide-based resin **R2** from the monomer 2-methoxy-6-[(4-vinyl)benzyloxy]benzylamine hydrochloride and synthesized the new resins **T3** and **T4** (Scheme 1) via immobilization onto commercial TentaGel S-Br of 2,6-dialkoxybenzylamine residues corresponding to molecules active as partially reversible BAO inhibitors with moderate (2,6-dimethoxybenzylamine, $IC_{50}(M) = 1.2 \times 10^{-4}$) or strong (2,6-diethoxybenzylamine, $IC_{50}(M) = 1.8 \times 10^{-7}$) inhibitory activity.¹⁷ For such immobilization, we modified the above inhibitor structures by introducing a linker at the benzene ring.

The poly(ethylene glycol) polystyrene graft copolymers TentaGel resins (TentaGel is a trademark of Rapp Polymere GmbH, 72072 Tübingen, Germany) were selected for the combination of the mechanical strength of their polystyrene backbones and the hydrophilicity of their polyoxyethylene grafts,^{20–24} which could be useful in possible column applications. Moreover, these resins, if properly functionalized, are known to interact with proteins and have been shown to be useful in the affinity capturing and purification of mammalian DNA polymerase- β .²⁵

With **T3** and **T4**, we wanted to open the way to new synthetic procedures of practical interest by exploiting a commercial resin, and

testing the effectiveness of a linker introduced in the position *para* to the aminomethyl group in a tetrasubstituted benzene ring that was rather easily obtained from symmetrical phloroglucinol. Two alkoxy groups providing substantially different inhibitory activity, aimed at forming CAOs complexes endowed with remarkably different stability, were selected in the view of obtaining easy blocking and release of CAOs, as required by the enzyme separation–purification protocols.

Essential and reliable tests of interaction between the prepared resins (**R2**, **T3**, **T4**) and commercial DAO or plasma amine oxidase (PAO), a CAO enzyme closely related to BAO, were performed through modified polyacrylamide gel electrophoresis (PAGE) experiments.

EXPERIMENTAL PROCEDURE

Materials

All the reagents and solvents were purchased from Sigma-Aldrich (Schnelldorf, Germany). The solvents were dried and distilled according to standard procedures. Petroleum ether refers to the fraction with a boiling point of 40–60 °C. 3,5-Diethoxyphenol,²⁶ 2,6-dimethoxy-4-hydroxybenzaldehyde (**3**),^{27,28} and (*E*)-1-chloro-4-(2-tetrahydropyran-2-yl)-2-butene (**11**)²⁹ were prepared according to known procedures. TentaGel S-Br (90 μ m, 0.26 mmol di Br per gram) was purchased from Fluka (Buchs, Switzerland).

PAO (22.5 U mg⁻¹, bovine plasma) was purchased from Worthington (Lakewood, NJ, USA), and DAO (0.18 U mg⁻¹, porcine kidney) was purchased from Sigma-Aldrich.

Characterization

The melting points, determined with a Leica Thermogalen III hot stage apparatus, and boiling points are uncorrected. The Fourier transform infrared (FTIR) spectra were recorded as films or KBr pellets using a PerkinElmer System 2000 spectrophotometer (PerkinElmer, Buckinghamshire, UK). The ¹H and ¹³C NMR spectra were acquired with a Bruker Avance DPX 300 spectrometer (Bruker BioSpin, Rheinstetten, Germany) at 300 and 75.5 MHz, respectively, using tetramethylsilane as the internal standard.

High pressure liquid chromatography (HPLC) analyses were performed at room temperature, constant flow rate (1 ml min⁻¹) and UV detection (254 nm) using a Merck LiChroart 125-4 HPLC cartridge (Merck, Darmstadt, Germany) or Hypersil ODS 5 μ m (25 $\times$ 0.46 cm), with a CH₃CN/H₂O = 60/40 mixture as the eluent except for compounds **1** and **2** for which a CH₃CN/H₂O = 80/20 mixture was necessary.

Chromatographic separations

Flash-chromatographic separations were performed on Merck Silica gel (0.040–0.063 mm). Thin layer chromatographies were obtained on Merck F₂₅₄ silica gel aluminum sheets. Preparative layer chromatographies were performed on Merck F₂₅₄ 60 silica gel plates (20 $\times$ 20 cm, 0.5 mm).

Scheme 1 Chemical structures of 4-(ω-aminoalkyl)phenyl polymers **P1a** and **P1c**, resin **R1a**, 2-methoxy-6-benzyloxybenzylamine resin **R2** and 2,6-dialkoxybenzylamine resins **T3** and **T4**.

Electrophoresis experiments

Procedure A without Sodium dodecyl sulfate (SDS) and β -mercaptoethanol (BME). The polyacrylamide gel for electrophoresis was composed of an 8% separating gel prepared in a 1.5-M Tris-HCl buffer (pH 8.8) with a 5% stacking gel prepared in a 0.5-M Tris-HCl buffer (pH 6.8).

The resins (0.6–3.5 mg) were swollen overnight in a 0.05-M borate/1.2-M urea buffer (pH 8.2, 50 μ l) and transferred to the wells. PAO (1 μ g) and DAO (10 μ g) from stock solutions (0.12–1.00 μ g μ l⁻¹), obtained by dissolving the enzyme in a 0.05-M borate/1.2-M urea buffer, were mixed with the resins and left to incubate in the well for 30 min before running the electrophoresis. A broad-range marker (10–260 kDa; Thermo Scientific, Rockford, IL, USA) was also loaded in parallel. Electrophoresis was performed in a Tris-glycine buffer (pH 8.5) at a current voltage range of 80–100 V for 3 h at 4 °C using a Bio-Rad Power Pack 300 (Bio-Rad Laboratories, Hercules, CA, USA). After the run, the gels were washed off the glass plates with a gel-fixing solution (50% ethanol and 10% acetic acid in water) and soaked in that solution for 30 min; the gels were then stained for 30 min in 0.1% Coomassie brilliant blue, 40% methanol and 10% acetic acid in water. To examine the protein bands, the gels were destained for 20 min (four times) and finally overnight in aqueous solutions containing 10% acetic acid and 20% methanol. After destaining, images of the gels were acquired using a digital scanner.

Further PAGE analyses were performed as described above with the following differences:

Procedure B in the presence of SDS. The separating and stacking gels were prepared as in procedure A with the addition of 10% SDS in both the gels; electrophoretic separation was performed in a Tris-glycine buffer (pH 8.5) containing SDS 0.1%.

Procedure C in the presence of SDS, BME and heating before loading into the gel. The separating and stacking gels were prepared as in procedure B. The enzyme solutions were added to the loading buffer (Tris-HCl solution with 2% SDS, 10% glycerol, 5% BME and bromophenol blue) and heated (10 min) at 95 °C before transferring into the gel; electrophoretic separation was then performed in a Tris-glycine buffer (pH 8.5) containing 0.1% SDS.

Synthesis

(*E*)-2,6-Dimethoxy-4-(4-hydroxy-2-butenyloxy)benzylamine hydrochloride (16**).** A suspension of LiAlH₄ (1.01 g, 26.6 mmol) in dry Et₂O (160 ml) was cooled to 0 °C under N₂, treated dropwise with a solution of (*E*)-2,6-dimethoxy-4-[(2-tetrahydropyranyloxy)-2-butenyloxy]benzonitrile (**12**) (Supplementary Information) (4.37 g, 13.1 mmol) in dry Et₂O (100 ml) at reflux for 3 h 30 min and left overnight at room temperature until disappearance of the infrared (IR) nitrile band at 2200 cm⁻¹. The mixture was hydrolyzed with 10% NaOH (300 ml), extracted with Et₂O (3 $\times$ 150 ml) and dried (Na₂SO₄).

The removal of the solvent at reduced pressure produced benzylamine **14** as an oil ((3.36 g, 76%), FTIR (film, cm⁻¹): 3371, 3306 (NH)), which was entirely converted into hydrochloride **16** by dissolution in a mixture of MeOH (49 ml) and H₂O (1 ml), and reaction at reflux with concentrated HCl (4.8 ml) under stirring and N₂ for 3 h. The removal of the solvent at reduced pressure yielded **16** as an oil that was crystallized from CH₃CN (1.66 g, 57%). Mp: 186–190 °C. Purity 95% by HPLC. FTIR (KBr, cm⁻¹): 3434 (OH + NH). ¹H-NMR (CD₃OD, δ p.p.m.): 3.86 (s, 6H); 4.07 (s, 2H); 4.08–4.12 (m, 2H); 4.58–4.63 (m, 2H); 5.86–6.06 (m, 2H); 6.29 (s, 2H). ¹³C-NMR: 33.24, 56.44, 62.83, 69.29, 92.36, 102.39, 126.61, 134.27, 160.91, 163.23.

(*E*)-2,6-Diethoxy-4-(4-hydroxy-2-butenyloxy)benzylamine hydrochloride (17**).** A suspension of LiAlH₄ (0.606 g, 16.0 mmol) in dry Et₂O (176 ml) was cooled to 0 °C under N₂, treated dropwise with a solution of (*E*)-2,6-diethoxy-4-[(2-tetrahydropyranyloxy)-2-butenyloxy]benzonitrile (**13**) (Supplementary Information) (2.84 g, 7.9 mmol) in dry Et₂O (89 ml) at reflux for 7 h 30 min and left overnight at room temperature until disappearance of the IR nitrile band at 2200 cm⁻¹. The mixture was hydrolyzed with 10% NaOH (170 ml), extracted with Et₂O (3 $\times$ 75 ml) and dried (Na₂SO₄).

The removal of the solvent at reduced pressure produced benzylamine **15** as an oil ((2.24 g, 77%); FTIR (film, cm⁻¹): 3377, 3312 (NH)), which was entirely

converted into hydrochloride **17** by dissolution in a mixture of MeOH (30 ml) and H₂O (0.7 ml), and reaction at reflux with concentrated HCl (3.0 ml) under stirring and N₂ for 3 h. The removal of the solvent at reduced pressure yielded **17** as an oil, which was crystallized from CH₃CN (1.03 g, 52%). Purity 99% by HPLC. Mp: 142–144 °C. FTIR (KBr, cm⁻¹): 3351 (OH + NH). According to NMR data, **17** consists of two slowly converting conformers. ¹H-NMR (CD₃OD, δ p.p.m.): 1.42 (minor, t, *J* = 7.0 Hz) and 1.43 (major t, *J* = 7.0 Hz), 6H); 4.01–4.14 (m, 1OH); 5.85–6.08 (m, 2H); 6.24 (s, 2H). ¹³C-NMR, major signals: 15.03, 33.40, 62.82, 65.35, 69.25, 93.05, 102.63, 126.64, 134.23, 160.19, 163.07; minor signals were observed at 65.15 and 93.38.

(*E*)-*N*-Triphenylmethyl-2,6-dimethoxy-4-(4-hydroxy-2-butenyloxy)benzylamine

(1). A solution of **16** (1.40 g, 4.8 mmol), dry CH₂Cl₂ (38 ml) and dry Et₃N (1.50 g, 14.8 mmol, 2 ml) was cooled to 0 °C and treated under N₂ with stirring with 4-(*N,N*-dimethylamino)pyridine (59.0 mg, 0.48 mmol) followed by solid triphenylmethyl chloride (1.75 g, 6.3 mmol). The solution was left under stirring at room temperature for 19 h, hydrolyzed with saturated NaHCO₃ (25 ml) and extracted with CH₂Cl₂ (3 $\times$ 25 ml). The extracts were combined, dried (Na₂SO₄) and concentrated at reduced pressure to produce an oily residue (3.37 g) that was crystallized from MeOH/H₂O = 9/2 to yield **1** (2.12 g, 90%). Purity 99% by HPLC. Mp: 86–90 °C. FTIR (KBr, cm⁻¹): 3419 (OH), 972 (CH = CH, *trans*), 706 (trityl). ¹H-NMR (CDCl₃, δ p.p.m.): 1.80 (s, broad, 2H, NH + OH); 3.20 (s, 2H); 3.72 (s, 6H); 4.17–4.24 (m, 2H); 4.50–4.56 (m, 2H); 5.89–6.03 (m, 2H); 6.11 (s, 2H); 7.14–7.59 (m, 15H). ¹³C-NMR: 36.52, 55.64, 62.86, 67.99, 71.02, 91.45, 110.32, 126.01, 126.12, 127.52, 129.06, 133.02, 146.44, 159.07, 159.24.

(*E*)-*N*-Triphenylmethyl-2,6-diethoxy-4-(4-hydroxy-2-butenyloxy)benzylamine

(2). A solution of **17** (0.744 g, 2.3 mmol), dry CH₂Cl₂ (18.5 ml) and dry Et₃N (0.708 g, 7.0 mmol, 0.98 ml) was cooled to 0 °C under N₂, stirred and then treated with 4-(*N,N*-dimethylamino)pyridine (28.1 mg, 0.23 mmol) followed by solid triphenylmethyl chloride (0.844 g, 3.0 mmol). The solution was left under stirring at room temperature for 24 h, hydrolyzed with saturated NaHCO₃ (14.9 ml) and extracted with CH₂Cl₂ (3 $\times$ 20 ml). The extracts were combined, dried (Na₂SO₄) and concentrated at reduced pressure to produce an oily residue (1.48 g), which was crystallized from MeOH to yield **2** (0.904 g, 74%). Purity 96% by HPLC. Mp: 75–77 °C. FTIR (KBr, cm⁻¹): 3422 (OH), 960 (CH = CH, *trans*), 706 (trityl). ¹H-NMR (CDCl₃, δ p.p.m.): 1.33 (t, 6H, *J* = 7.0 Hz); 1.70 (s, broad, NH + OH); 3.25 (s, 2H); 3.93 (q, 4H, *J* = 7.0 Hz); 4.17–4.22 (m, 2H); 4.48–4.54 (m, 2H); 5.89–6.02 (m, 2H); 6.09 (s, 2H); 7.14–7.61 (m, 15H). ¹³C-NMR: 15.04, 36.57, 62.90, 63.84, 67.95, 71.01, 92.05, 110.27, 125.97, 126.22, 127.55, 129.07, 132.96, 146.50, 158.71, 158.91.

Resins T1 and T2. TentaGel S-Br (0.50 g, 90 μ m, 0.26 mmol Br per gram) was left to swell in dry *N,N*-dimethylformamide (DMF) (3 ml) at room temperature for 80 min, cooled to 0 °C, treated with a solution of the sodium salt of benzylamines **1** or **2** (0.26 eq.) in DMF (3 ml) and then heated at 88 °C for 18 h. The resin was filtered and washed with DMF (4 ml), DMF/H₂O 1/1 (4 ml), THF/H₂O 1/1 (4 ml), MeOH/H₂O 1/1 (4 ml) and MeOH 100% (4 ml), filtered, and brought to constant weight at reduced pressure. **T1** (0.48 g); **T2** (0.47 g).

Detritylation of T1 and T2. Resin **T1** (or **T2**) (0.40 g) was left to swell under N₂ and magnetic stirring in dry THF (6 ml) for 30 min, treated with concentrated HCl (0.25 ml) at reflux for 16 h, cooled and left overnight under stirring in excess MeOH. The resin was filtered, washed repeatedly with dry THF, filtered and brought to constant weight at reduced pressure. **T3** (0.39 g); **T4** (0.36 g).

RESULTS AND DISCUSSION

Resin **R2** containing the monomers 2-methoxy-6-[(4-vinyl)benzyloxy]benzylamine hydrochloride and *N,N*-dimethylacrylamide, with *N,N'*-ethylenedisacrylamide as the crosslinker, was prepared in deoxygenated water in the presence of the radical initiator ammonium persulphate as previously reported.¹⁹ The process

followed in every aspect what was already observed. The obtained resin **R2** had a loading of NH_2 groups = $311 \mu\text{mol g}^{-1}$.

New polar stationary phases were synthesized by anchoring 2,6-dialkoxybenzylamine residues through stable ether bonds onto commercial TentaGel S-Br, which contains bromomethyl groups susceptible to nucleophilic substitution reactions.

The 2,6-dialkoxybenzylamine residues were introduced on the resin in the form of the *N*-trityl-protected precursors **1** and **2** (Scheme 2). The 4-hydroxy-2-butenyloxy spacer with *E* stereochemistry was selected to facilitate both the anchoring of **1** and **2** to the bulky resin and the successive interaction with the enzymes.

Synthesis of benzylamine derivatives **1** and **2**

The benzylamine derivatives **1** and **2** were prepared according to Scheme 3, starting from the known benzaldehyde **3**^{27,28} or the new benzaldehyde **4** obtained through the Vilsmeier formylation of 3,5-diethoxyphenol²⁶ (Supplementary Information).

After considering the various methodologies used to convert aldehydes into nitriles and the presence of phenolic OH, the benzaldehydes **3** and **4** were transformed into **9** and **10** through the mild neutral dehydration of oximes **7** and **8** catalyzed by $\text{Cu}(\text{OAc})_2$ ³⁰ however, the reaction failed occasionally with oxime **7**. Alternative

reagents, such as DMF/ 135°C ³¹ and Ac_2O ³² used to promote the dehydration of **7** did not yield substantial improvements.

The nitriles **9** and **10** were alkylated with (*E*)-1-chloro-4-(2-tetrahydropyranyloxy)-2-butene (**11**)²⁹ to yield **12** and **13** as oils, which after purification through column chromatography also yielded variable quantities of the corresponding alcohols without the THP residue, indicating a certain instability of the protecting group to silica. The nitriles **12** and **13** were converted into hydrochlorides **16** and **17** after reduction with lithium aluminum hydride followed by acid treatment to remove the tetrahydropyranyl groups. The desired benzylamines **1** and **2** were finally obtained by reaction with trityl chloride in the presence of 4-(*N,N*-dimethylamino)pyridine.

Functionalization of TentaGel S-Br with **1** and **2**

The functionalization of commercial TentaGel S-Br was performed by swelling the resins in DMF, treating them with the sodium alkoxides of *N*-trityl benzylamines **1** and **2**, and removing the trityl protection by reaction with concentrated HCl (Scheme 4).

The content of NH_2 groups of the new resins was determined to be $13 \mu\text{mol g}^{-1}$ for **T3** and $7 \mu\text{mol g}^{-1}$ for **T4**; these contents were determined according to the method of Gaur and Gupta,³³ which is based on the introduction of 4,4'-dimethoxytrityl residues to the NH_2 groups and spectrophotometric determination of the 4,4'-dimethoxytrityl cation ($\epsilon = 70,000$ at 498 nm) obtained after treating the resin with HClO_4 . These values indicate a difficult functionalization with low transformation yields of the starting resins even though a temperature higher than those used in other TentaGel S-Br alkylations^{34,35} was applied. Nevertheless, the degrees of functionalization appeared suitable to perform the interaction tests, considering that for efficient biorecognitions a low ligand density is often required in affinity chromatography.³⁶

Enzyme–resins interactions

To determine the capability of resins **T3**, **T4** and **R2** in capturing enzymes of the CAO family, we used PAGE experiments, which consumed very small quantities of both enzymes and resins, and offered rapid responses.

Scheme 2 *N*-Trityl-2,6-dialkoxybenzylamine derivatives **1** and **2** and their aldehyde precursors **3** and **4**.

Scheme 3 Synthesis of benzylamines **1** and **2**.

Scheme 4 Introduction of 2,6-dialkoxybenzylamine residues onto Tentagel S-Br. a, NaH/DMF/88 °C; b, Tentagel S-Br; c, HCl concentrated/THF/reflux.

Figure 1 (a) Without SDS or BME. Lane 1: molecular weight (MW) marker (kDa); lane 2: PAO alone; lane 3: PAO + Tentagel S-Br; lane 4: PAO + **T3**; lane 5: PAO + **T4**; lane 6: PAO + **R2**; lane 7: DAO alone; lane 8: DAO + **T3**; lane 9: DAO + **T4**; lane 10: DAO + **R2**. (b) In the presence of SDS. Lane 1: MW marker (kDa); lane 2: PAO alone; lane 3: PAO + Tentagel S-Br; lane 4: PAO + **T3**; lane 5: PAO + **T4**; lane 6: PAO + **R2**; lane 7: DAO alone; lane 8: DAO + **T3**; lane 9: DAO + **T4**; lane 10: DAO + **R2**.

Figures 1 and 2 illustrate typical electrophoretic runs of the PAGE experiments with the enzymes PAO and DAO. The amount of enzyme charged in each lane for each enzyme set was the same to discern possible differences after the runs.

The experiments were performed either following the Laemmli protocol³⁷ by adding SDS and BME as the reducing agent with heating (denaturing conditions), or by adding SDS only or in the absence of SDS and BME.

In the absence of SDS and BME, PAO and DAO exhibited two spots corresponding to homodimer and subunit, respectively, when the enzymes were alone (Figure 1a; lanes 2 and 7) and also when they were with Tentagel S-Br or its chemical modifications **T3** and **T4** (Tentagels) (Figure 1a; lanes 3, 4, 5 and 8, 9). Under these conditions, PAO and DAO with **R2** (Figure 1a; lanes 6 and 10) produced lanes completely free from spots.

In the presence of SDS, PAO either alone (Figure 1b; lane 2) or with Tentagels (Figure 1b; lanes 3, 4, 5) exhibited only the subunit spot, with the relevant fact that in the cases of PAO with all the Tentagels, an appreciably lower mass that could correspond to the loss of the saccharide portion of the subunit was observed. DAO either alone (Figure 1b; lane 7) or with modified Tentagels (Figure 1b; lanes 8, 9) exhibited two spots corresponding to the homodimer and subunit, respectively; however, with the Tentagels, the subunit spots had a mass lower than that of DAO alone, in analogy to what was observed for the PAO subunit. Evidently, the Tentagels matrices under the reported conditions were not inactive toward the enzyme.

In the presence of SDS, PAO and DAO with **R2** (Figure 1b; lanes 6 and 10) produced lanes free from spots.

Figure 2 In the presence of SDS, BME and heating. Lane 1: molecular weight (MW) marker (kDa); lane 2: PAO alone; lane 3: PAO + Tentagel S-Br; lane 4: PAO + **T3**; lane 5: PAO + **T4**; lane 6: PAO + **R2**; lane 7: DAO alone; lane 8: DAO + **T3**; lane 9: DAO + **T4**; lane 10: DAO + **R2**.

Despite the lack of a perfect matching of molecular weights, it was evident that either in the absence or presence of SDS, the Tentagels did not capture PAO and DAO, while resin **R2** captured both PAO and DAO.

The observed coincident behavior of the unmodified and modified Tentagels demonstrated that the chemical modifications performed to obtain **T3** and **T4** did not yield materials with the desired bioactivity.

In the presence of SDS and BME with heating, under denaturing conditions that excluded the presence of the homodimer and allowed for a better matching of the molecular weights, the electrophoretic

runs with **R2** (Figure 2; lanes 6 and 10) exhibited the spots of PAO and DAO subunits together with another two spots at lower mass, presumably originating from the enzyme fragmentation for DAO. The fact that **R2** did not capture the denaturated enzymes, while capturing PAO and DAO in non-denaturing conditions, confirmed that the substrate-like inhibitor residues present in the resin were able to form stable complexes with the CAOs. In this manner, the residues allowed **R2** to be the first synthetic insoluble polymeric material to stably capture CAOs onto a resin.

The resins **T3** and **T4**, first attempts of using Tentagel resins modified for capturing CAOs, failed. Nevertheless, the results suggest that new explorations with differently structured bioactive functions based on trisubstituted benzene and pyridine rings, preferentially anchored on a matrix less active than TentaGel S-Br, if available, may be successful.

CONCLUSIONS

We synthesized the resins **R2**, **T3** and **T4** containing different substrate-like inhibitor-benzylamine structures designed to interact with CAOs. After several optimizations, the recommended procedure for the synthesis of **T3** and **T4** was performed through the seven-step synthesis of 2,6-dialkoxybenzylamine derivatives **1** and **2**, exploiting the Vilsmeier formylation of 3,5-dimethoxy and 3,5-diethoxyphenol, respectively, followed by transformation of the formyl group into nitrile, reduction and *N*-protection of the NH₂ group with trityl residues. **1** and **2** were then anchored onto commercial TentaGel S-Br resin.

We established simple and reliable tests of interaction between the prepared resins (**R2**, **T3**, **T4**) and CAOs based on PAGE experiments in the absence and presence of SDS and also with BME.

We observed that the chemical modifications of the commercial TentaGel S-Br resin did not yield the desired bioactive materials and that such a commercial matrix in the presence of SDS caused denaturation of PAO and DAO, and lowered the mass of their subunits, which could correspond to the loss of the saccharide portion.

We observed that resin **R2** was the first synthetic insoluble polymeric material able to capture enzymes of the CAO family through the formation of stable complexes.

Our findings suggest further explorations to immobilize new functions onto inactive commercial resins using bioactive trisubstituted benzene or pyridine rings as substrate-like inhibitors.

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