Available online at www.sciencedirect.com
SCIENCE DIRECT
Tetrahedron
ELSEVIER
Tetrahedron 60 (2004) 11407–11414

Monomers containing substrate or inhibitor residues for copper amine oxidases and their hydrophilic beaded resins designed for enzyme interaction studies

Vincenzo Bertini,[a,*] Silvana Alfei,[a] Marco Pocci,[a] Francesco Lucchesini,[a] Nevio Picci[b] and Francesca Iemma[b]

[a]*Dipartimento di Chimica e Tecnologie Farmaceutiche e Alimentari, Università di Genova, Via Brigata Salerno, I-16147 Genova, Italy*
[b]*Dipartimento di Scienze Farmaceutiche, Università della Calabria, I-87030 Arcavacata di Rende, Italy*

Received 10 June 2004; revised 1 September 2004; accepted 23 September 2004

Available online 12 October 2004

Abstract—Five styrenic monomers, four with aminoalkyl residues typical of copper containing amine oxidase substrates and one with a 2,6-dialkoxybenzylamine residue which mimics previously prepared selective substrate-like benzylamine oxidase inhibitors, have been synthesized and transformed into radical homopolymers, copolymers with *N*,*N*-dimethylacrylamide (DMAA), and hydrophilic beaded resins, designed for enzyme interaction studies aimed in finding new materials for highly biospecific chromatographic separations. The five monomers have given beaded resins of 125–500 μm swellable in water with a volume increase of 1200–1500%. The four aminoalkyl monomers have given water soluble copolymers some of which are good substrates of benzylamine oxidase (BAO), diamine oxidase (DAO) and lysyl oxidase (LO), up to 9.7 times better than elastin for LO.

1. Introduction

Copper amine oxidases (CAOs)[1] are a large family of enzymes (EC 1.4.3.6) present in prokaryotes and eukaryotes including man, that control important cellular processes such as the removal of biogenic amines, the cross-linking of elastin and collagen, the regulation of intracellular polyamines. The enzymatic reaction consists of the oxidative deamination of aminomethyl substrates to produce aldehydes, hydrogen peroxide and ammonia. In the last decade, X-ray structure determinations[2] of CAOs obtained from microorganisms and plants have allowed important progress in the knowledge of cofactors,[3] enzymatic reaction mechanism,[4] role of the copper etc. evidencing significant differences among them. Nevertheless, no crystallographic data are available at present for the mammalian members of CAOs that suffer from laborious purification protocols.[5–8]

With the final aim of improving and shortening the enzyme purification procedures through the synthesis of new materials for biospecific chromatographic separations, we want to report in this work the preparation and characterization of highly hydrophilic *N*,*N*-dimethylacrylamide-based resins of the type **R1** and **R2** (Fig. 1), both conceived as macromolecular tools for biospecific interactions with mammalian CAOs. The structure of **R1** is characterized by aminoalkyl residues typical of the CAO substrates, while **R2** contains 2,6-dialkoxybenzylamine residues which mimic previously prepared selective benzylamine oxidase inhibitors[9] with IC_{50}(M) up to 6.6×10^{-8}.

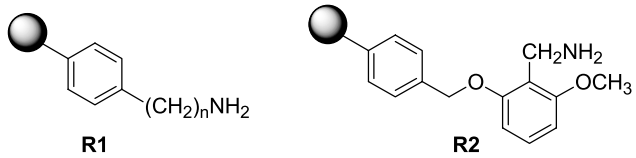


Figure 1.

The preparation of macromolecular systems for specific interactions with enzymes usually active on small molecule substrates is expected to be quite a demanding task that requires the careful choice and optimization of many parameters such as type of the active functions, nature and length of the linker, physical and chemical stability, degree of hydrophilicity, flow properties and accessibility of the

Keywords: Monomers; Polymers; Hydrophilic beaded resins; Copper amine oxidases; Reverse suspension polymerization.
* Corresponding author. Tel.: +39 010 353 2685; fax: +39 010 353 2684; e-mail: bertini@dictfa.unige.it

0040–4020/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2004.09.083

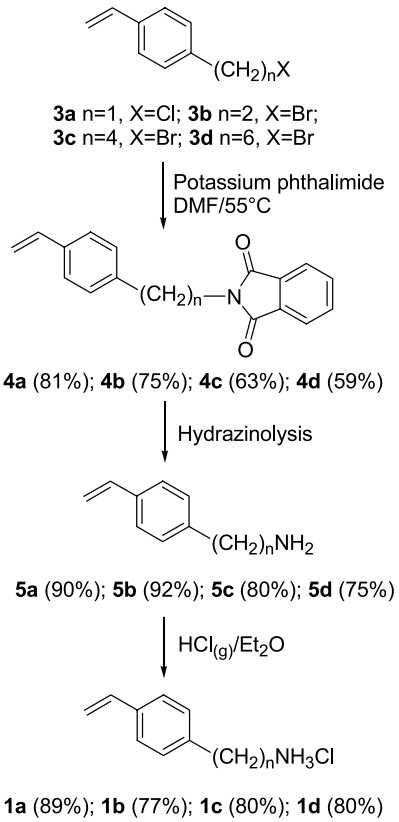
active functions. Consequently, we rejected synthetic procedures entailing functionalization of preformed commercial matrices[10] preferring the synthesis ab initio of functionalized monomers and copolymers to gain advantages such as the control of structure, purity, and loading of the active monomer, and flexibility in tailoring important properties for the interaction process with the enzyme such as the swellability and porosity of the macromolecular system.[11]

2. Results and discussion

2.1. Synthesis of substrate-like monomers 1a–d

Monomers **1a–d** were synthesized according to the Scheme 1. The necessary ω-haloalkylstyrenes were purchased (**3a**), or obtained through the copper halide coupling reaction[12,13] of α,ω-dibromoalkanes with styryl Grignard reagents[14] with minor modifications[15] (**3c** and **3d**), or prepared (**3b**)[16] by an alternative route based on the acylation of 2-bromoethylbenzene, since 1,2-dibromoethane forms ethylene when it reacts with Grignard reagents.[13] The purification of **3c** and **3d** was preferentially performed by column chromatography to avoid occasional polymerization during distillation at reduced pressure.

The successive reaction of ω-haloalkylstyrenes with potassium phthalimide in DMF afforded Gabriel adducts **4a–d** which were purified and characterized before submission to hydrazinolysis. The obtained ω-aminoalkylstyrenes **5a–d** were promptly transformed into the corresponding hydrochlorides **1a–d** for better purification and storage.

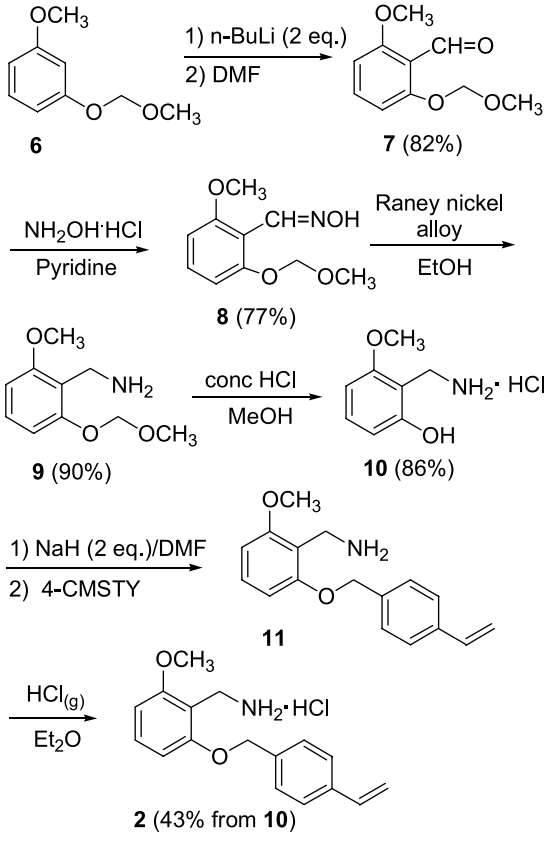


Scheme 1.

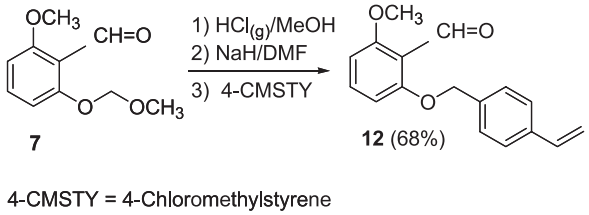
2.2. Synthesis of the inhibitor-like monomer 2

The multistep synthesis of **2**, performed according to Scheme 2, is based on the *ortho*-directed metallation of 3-methoxymethoxyanisole (**6**)[17] for which we recently highlighted some very interesting results, producing the desired aldehyde in good yield, or substituting the methoxymethoxy moiety with formation of tri- and tetrasubstituted benzenes, or affording quite unusually stable doubly lithiated intermediates.[18] The aldehyde **7**, after transformation into its oxime **8** and reduction, afforded **9** as an easily distillable liquid. The deprotection of **9** afforded **10** in good yield, but the successive alkylation with 4-chloromethylstyrene to produce the crude free base **11**, then monomer **2** with an overall 26% yield from **7** was not as good.

Following an alternative route (Scheme 3), **7** was transformed into 2-methoxy-6-hydroxybenzaldehyde[19] which was alkylated with 4-chloromethylstyrene to afford 2-methoxy-6-[(4-vinyl)benzyloxy]benzaldehyde (**12**) in good yield, but **12** proved difficult to transform into **11** either by reducing its oxime derivative with the Raney nickel alloy or by treating it with sodium cyanoborohydride and ammonium acetate. If **11** in the form of its hydrochloride **2** is demonstrated to be very effective in the



Scheme 2.



Scheme 3.

biological tests, its synthesis and the obstacles to the accomplishment of its structure will be further examined.

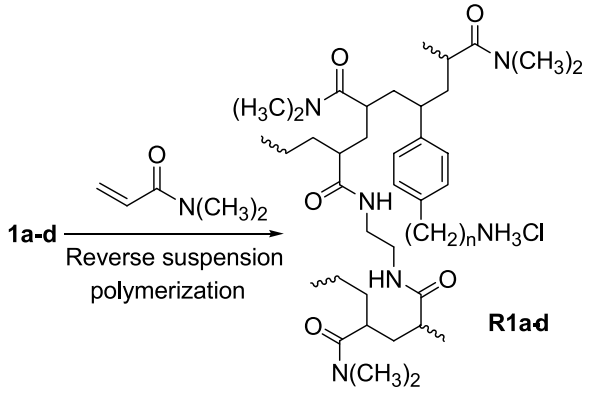
2.3. Polymers and resins

Preliminary polymerization studies showed that the monomers **1a–d** and **2** homopolymerized and copolymerized easily with *N*,*N*-dimethylacrylamide, a good hydrophilic comonomer for polar supports,[20] in water with ammonium persulfate and in methanol or DMF with AIBN as radical initiators affording conversions in the range 20–94%. In the IR spectra of the homopolymers, intense broad absorptions around 3000 cm^{-1} due to NH_3^+ groups were present, while the IR spectra of the copolymers showed the amide band of the comonomer around 1620 cm^{-1}.

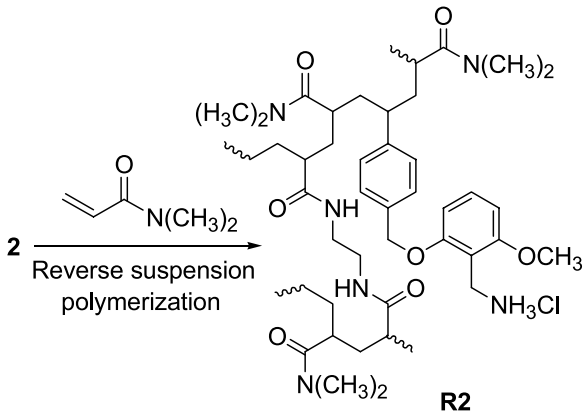
All the copolymers with DMAA were soluble in water and methanol and insoluble in petroleum ether, benzene, diethyl ether, dioxane, acetone. DMF proved to be a good solvent for polymers containing units of **2** and a poor solvent for those containing units of **1a–d**.

Preliminary biological tests showed that some of the prepared polymeric materials were good to excellent substrates for different CAOs.

After these positive indications, the transformation of **1a–d** and **2** into cross-linked resins was achieved by suspending the aqueous solution of the monomers in a mixture of CCl_4/hexane and using SPAN 85 and APS/TMEDA as anticoagulant and initiator respectively[21] (Schemes 4 and 5).



Scheme 4.



Scheme 5.

The conditions applied afforded very good conversions (92–95%) for all the monomers except for **1d** (51%). Probably, the longer hydrophobic methylene sequence in **1d** makes it more soluble in the organic phase thus slowing the polymerization process. This fact and other undesired properties of the resins **R1d** (see below) made us lose interest in this monomer.

The resins **R1a–c** and **R2**, with the exclusion of **R1d**, appear as microspherular beads with the bulk of material (>96%) in the size range 125–500 μm. They are endowed with high hydrophilicity, as estimated by observing the increase of volume of the dry material after overnight swelling in water (Table 1), and show good flow properties. Fig. 2 shows a typical sample of a dry and a swollen resin.

The NH_2 content of the resins was estimated following the method of Gaur and Gupta[22] based on the labeling of the amino groups with 4-O-(4,4′-dimethoxytriphenylmethyl)-butyryl residues and the quantitative determination through UV-Vis spectroscopy of the 4,4′-dimethoxytriphenylmethyl cation ($\varepsilon = 70{,}000$ at 498 nm) released from the resin after treatment with $HClO_4$. The values of NH_2 loading (Table 1) for all the prepared resins appear to be suitable for enzyme interaction studies, which for their specificity deserve a separate paper. Nevertheless, keeping in mind that among CAOs only lysyl oxidase (LO) is naturally devoted to oxidize lysine residues in macromolecular structures like elastin and collagen while all the other enzymes of the same class oxidize small molecules, some remarkable results highlighting the bioactivity levels of some of our polymeric materials are briefly anticipated in Table 2.

The soluble copolymers **P1a** and **P1c**, and resin **R1a** are very good substrates of LO and benzylamine oxidase (BAO), with **P1c** also active towards diamine oxidase (DAO). As far as LO is concerned, it is noteworthy that resin **R1a** is as active as the natural substrate elastin, and copolymer **P1c** is 9.7 times more active than the elastin itself.

3. Conclusions

Since the first essential step for setting up a biospecific separation of CAOs is the synthesis of polymeric materials

Table 1. Data of some representative reverse-phase suspension copolymerizations of **1a–c** and **2** with *N,N*-dimethylacrylamide and *N,N′*-ethylenebisacrylamide

Monomers g (mole fraction)	H_2O mL	Span 85 µl	CCl_4/Hexane mL/mL	APS g	TMEDA µl	Time min	Resin, g (%)	Volume Increase[a]%	NH_2 µmol/g
1a 2.96 (0.140) DMAA 9.91 (0.799) EBA 1.29 (0.061)	89	521	154/264	0.25	510	90	**R1a**, 12.98 (92)	1160	696
1b 3.00 (0.187) DMAA 6.47 (0.748) EBA 0.95 (0.065)	120	680	215/360	0.22	780	105	**R1b**, 9.54 (92)	1520	932
1c 4.08 (0.138) DMAA 12.3 (0.797) EBA 1.72 (0.065)	122	680	205/360	0.34	680	90	**R1c**, 17.84 (98)	1260	894
2 1.00 (0.138) DMAA 1.80 (0.762) EBA 0.40 (0.100)	H_2O/DMF, (1.2/1.0), 34	110	64/128	0.16	320	120	**R2**, 3.21 (99)	1340	305

[a] Calculated from the formula $100(V_s - V_d)/V_d$ where V_s and V_d are the volumes of the swollen and dry resin, respectively.

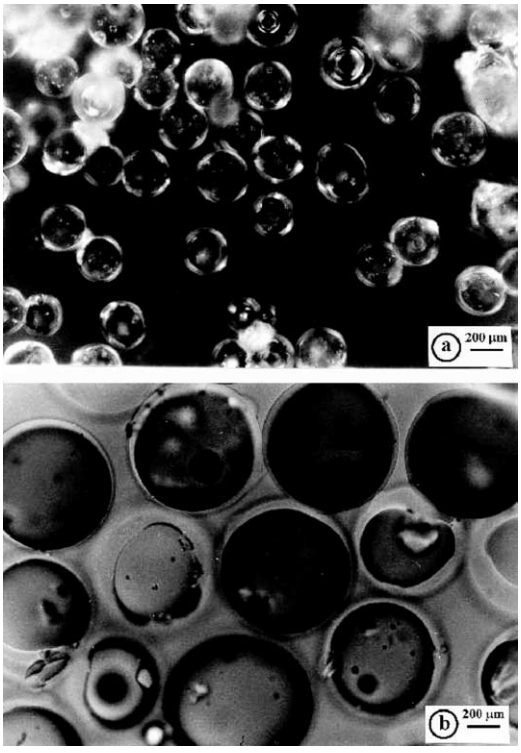


Figure 2. Optical microphotographs of resin **1b**/DMAA/EBA: (a) dry; (b) swollen in water.

Table 2. Substrate activities of some of the prepared polymeric materials as percentage of the activity of the best substrate for each enzyme

Copolymer or resin	Enzyme (substrate)	Substrate activity (%)
P1a	LO (a)	64
P1a	BAO (b)	63
P1c	LO (a)	970
P1c	BAO (b)	89
P1c	DAO (c)	30
R1a	LO (a)	100
R1a	BAO (b)	62

Enzymes: LO=lysyl oxidase from porcine aorta; BAO=benzylamine oxidase from porcine serum; DAO=diamine oxidase from porcine kidney. Best substrates: a=elastin; b=benzylamine; c=puterescine.

able to interact with the enzyme active site either as substrate or as reversible not denaturing inhibitor, we prepared four styrenic monomers with aminoalkyl residues (**1a–d**) having alkyl chains of different length designed as substrates and one styrenic monomer with a 2,6-dialkoxybenzylamine residue (**2**) designed as inhibitor.

The four monomers **1a–d** were transformed into soluble copolymers and microspherular water swellable resins with DMAA as comonomer, rejecting the materials from **1d** due to inadequate properties. Copolymers and resins obtained from **1a** and **1c** proved reactive as substrate of LO, BAO and DAO beyond all expectations.

For the monomer **2** which mimics selective substrate-like inhibitor of BAO, a multistep synthesis, based on the formylation of 3-methoxymethoxyanisole and flexible to structural modifications regarding type and length of the

linker, was performed. Monomer **2** easily afforded after copolymerization with DMAA microspherular beaded swellable resins whose biological behavior with CAOs is under study.

4. Experimental

4.1. Instruments and methods

Melting points and boiling points are uncorrected. FTIR spectra were recorded as films or KBr pellets on a Perkin Elmer System 2000 instrument. ^{1}H and ^{13}C-NMR spectra were acquired on a Bruker DPX spectrometer at 300 and 75.5 MHz respectively with tetramethylsilane as internal reference. Mass spectra were obtained with a GC-MS Ion Trap Varian Saturn 2000 instrument (EI or CI mode; filament current: 10 μA) equipped with a DB-5MS (J&W) capillary column. UV–Vis spectra were recorded with a Varian Cary 18 spectrometer. Microanalyses were obtained from the Laboratorio di Microanalisi (Faculty of Pharmacy, University of Pisa).

HPLC analyses were performed at room temperature, constant flow rate (1 mL/min) and UV detection (254 nm) using a 25×0.46 cm Hypersil ODS 5 μm column using a mixture acetonitrile/water=6/4 as eluent. GC-FID analyses were performed on Perkin Elmer Autosystem using a DB-5, 30 m, i.d. 0.32 mm, film 1 μm capillary column. Column chromatographies were performed on Merck silica gel (70–230 mesh). TLCs were obtained on Merck F_{254} silica gel plates.

Optical microphotographs were obtained with a Zeiss Axioskop instrument. Sieving was performed with a 2000 Basic Analytical Sieve Shaker-Retsch apparatus.

4.2. Materials

4-Chloromethylstyrene (**3a**) and all the other reagents and solvents were from Sigma-Aldrich and were purified by standard procedures. Azobisisobutyronitrile (AIBN) was crystallized from methanol. 4-(2-Bromoethyl)styrene (**3b**),[16] *N*,*N*′-ethylenebisacrylamide[20] (EBA), and *N*-succinimidyl-4-O-(4,4′-dimethoxytriphenylmethyl)butyrate[23] were prepared by known procedures.

Further acronyms and registered trademarks of commercial products used are: APS=ammonium persulfate; DMAA=*N*,*N*-dimethylacrylamide; DMF=*N*,*N*-dimethylformamide; TMEDA=*N*,*N*,*N*′,*N*′-tetramethylethylenediamine; SPAN 85=sorbitan trioleate.

4.3. 4-(ω-Bromoalkyl)styrenes 3c and 3d

A mixture of the α,ω-dibromoalkane (50 mmol), dry THF (20 mL) and a solution of $LiCuBr_2$[15] (1.5 mL) in dry THF was cooled to 0 °C, treated dropwise with 0.68 M 4-vinylphenyl magnesium chloride (18 mL, 12.2 mmol) in THF and stirred at room temperature for 5 h. The reaction mixture was then treated with an iced aqueous solution of NaCN (0.80 g) and NH_4Cl (5.00 g) dissolved in water (35 mL) and extracted with peroxide-free ethyl ether (4×30 mL). The extracts were dried over anhydrous $MgSO_4$, treated at reduced pressure to remove most of the unreacted dibromide and purified by column chromatography using petroleum ether 40–60 °C as eluent to afford monomers **3c** and **3d** as colorless liquids.

4.3.1. 4-(4-Bromobutyl)styrene (3c). 1.87 g (64%); bp=100 °C/0.15 torr, [lit.[24]: 92–93 °C/0.1 torr]. IR (film, ν, cm^{-1}) 990, 906 (CH_2=CH). ^{1}H NMR ($CDCl_3$, ppm) 1.76 (m, 2H); 1.86 (m, 2H); 2.61 (t, 2H, J=7.4 Hz); 3.39 (t, 2H, J=6.6 Hz); 5.19 (dd, 1H, J_1=1.0 Hz, J_{cis}=10.9 Hz); 5.70 (dd, 1H, J_1=1.0 Hz, J_{trans}=17.6 Hz); 6.68 (dd, 1H, J_{cis}=10.9 Hz, J_{trans}=17.6 Hz); 7.11–7.34 (m, 4H). ^{13}C NMR 29.74; 32.18; 33.60; 34.66; 113.05; 126.24; 128.54; 135.36; 136.61; 141.50. GC-MS (EI, *m*/*z*, %): 240 (M^+ [^{81}Br], 37); 238 (M^+ [^{79}Br], 34); 117 (100).

4.3.2. 4-(6-Bromohexyl)styrene (3d). 2.25 g (77%); bp=80 °C/0.06 torr. IR (film, ν, cm^{-1}) 990, 905 (CH_2=CH). ^{1}H NMR ($CDCl_3$, ppm) 1.29–1.51 (m, 4H); 1.62 (m, 2H); 1.85 (m, 2H); 2.59 (t, 2H, J=7.5 Hz); 3.39 (t, 2H, J=6.8 Hz); 5.18 (dd, 1H, J_1=1.0 Hz, J_{cis}=10.9 Hz); 5.70 (dd, 1H, J_1=1.0 Hz, J_{trans}=17.6 Hz); 6.69 (dd, 1H, J_{cis}=10.9 Hz, J_{trans}=17.6 Hz); 7.11–7.34 (m, 4H). ^{13}C NMR 28.02; 28.34; 31.14; 32.73; 33.88; 35.51; 112.88; 126.17; 128.55; 135.18; 136.71; 142.32. GC–MS (EI, *m*/*z*, %): 268 (M^+ [^{81}Br], 59); 266 (M^+ [^{79}Br], 57); 117 (100).

4.4. *N*-[(4-Vinylphenyl)alkyl]phthalimides 4a–d

A mixture of 4-haloalkylstyrene (**3a–d**) (26.6 mmol), potassium phthalimide (27.4 mmol) and dry DMF (25 mL) was heated at 55 °C under nitrogen and mechanical stirring for 17 h. After removal of the solvent at reduced pressure the solid residue was taken with chloroform (40 mL), filtered and washed with chloroform (3×10 mL). All the organic extracts were combined, washed with 0.2 M NaOH (15 mL), water (2×15 mL) and dried over anhydrous Na_2SO_4. The removal of the solvent at reduced pressure afforded **4a–d** as a crude solid which was crystallized from methanol (**4a–b**) or column chromatographed using benzene as eluent.

4.4.1. *N*-(4-Vinylbenzyl)phthalimide (4a). Reagent **3a**. Yield 81%. White flakes. Mp 107 °C; [lit.[25]: 107–108 °C]. Purity 99% by HPLC. IR (KBr, ν, cm^{-1}) 1704 (C=O), 995, 914 (CH_2=CH). ^{1}H NMR ($CDCl_3$, ppm) 4.82 (s, 2H); 5.21 (dd, 1H, J_{gem}=0.9 Hz; J_{cis}=10.9 Hz); 5.70 (dd, 1H, J_{gem}=0.9 Hz; J_{trans}=17.6 Hz); 6.66 (dd, 1H, J_{cis}=10.9 Hz; J_{trans}=17.6 Hz); 7.36 (m, 4H); 7.67–7.82 (m, 4H). ^{13}C NMR 41.31, 114.13, 123.31, 126.47, 128.84, 132.10, 133.96, 135.86, 136.31, 137.18, 167.98. GC-MS (CI, *m*/*z*, %): 264 (M^++1, 100). Anal. Calcd. for $C_{17}H_{13}NO_2$: C, 77.55; H, 4.98; N, 5.32. Found: C, 77.32; H, 5.00; N, 5.32.

4.4.2. *N*-[2-(4-Vinylphenyl)ethyl]phthalimide (4b). Reagent **3b**. Yield 75%. White flakes. Mp 135–137 °C. Purity 98% by HPLC. IR (KBr, ν, cm^{-1}) 1703 (C=O), 990, 907 (CH_2=CH). ^{1}H NMR ($CDCl_3$ ppm) 2.98 (t, 2H, J=7.8 Hz); 3.91 (t, 2H, J=7.8 Hz); 5.20 (dd, 1H, J_{gem}=1.0 Hz; J_{cis}=10.9 Hz); 5.70 (dd, 1H, J_{gem}=1.0 Hz; J_{trans}=17.6 Hz); 6.67 (dd, 1H, J_{cis}=10.9 Hz; J_{trans}=17.6 Hz); 7.21–7.32 (m, 4H); 7.69–7.82 (m, 4H). ^{13}C NMR 34.28,

39.14, 113.42, 123.22, 126.40, 129.01, 132.06, 133.90, 136.01, 136.54, 137.63, 168.14. GC-MS (CI, *m/z*, %): 278 ($M^+ + 1$, 100). Anal. Calcd. for $C_{18}H_{15}NO_2$: C, 77.96; H, 5.45; N, 5.05. Found: C, 77.86; H, 5.47; N, 5.04.

4.4.3. *N*-[4-(4-Vinylphenyl)butyl]phthalimide (4c). Reagent **3c**. Yield 63%. White grains. Mp 117–119 °C. Purity 98% by HPLC. IR (KBr, ν, cm^{-1}) 1703 (C=O), 992, 913 (CH_2=CH). ^1H NMR ($CDCl_3$ ppm) 1.69 (m, 4H); 2.64 (t, 2H, J=7.0 Hz); 3.71 (t, 2H, J=7.0 Hz); 5.18 (dd, 1H, J_{gem}=1.0 Hz; J_{cis}=10.9 Hz); 5.69 (dd, 1H, J_{gem}=1.0 Hz; J_{trans}=17.6 Hz); 6.68 (dd, 1H, J_{cis}=10.9 Hz; J_{trans}=17.6 Hz); 7.13–7.36 (m, 4H); 7.67–7.85 (m, 4H). ^{13}C NMR 28.14, 28.53, 35.07, 37.76, 112.94, 123.18, 126.20, 128.59, 132.14, 133.87, 135.27, 136.66, 141.73, 168.43. GC-MS (EI, *m/z*, %): 305 (M^+, 100). Anal. Calcd. for $C_{20}H_{19}NO_2$: C, 78.66; H, 6.27; N, 4.59. Found: C, 78.62; H, 6.26; N, 4.58.

4.4.4. *N*-[6-(4-Vinylphenyl)hexyl]phthalimide (4d). Reagent **3d**. Yield 59%. White needles. Mp 75–77 °C. Purity 99% by HPLC. IR (KBr, ν, cm^{-1}) 1707 (C=O), 988, 966 (CH_2=CH). ^1H NMR ($CDCl_3$, ppm) 1.30–1.72 (m, 8H); 2.58 (t, 2H, J=7.5 Hz); 3.67 (t, 2H, J=7.5 Hz); 5.18 (dd, 1H, J_{gem}=1.0 Hz; J_{cis}=10.9 Hz); 5.69 (dd, 1H, J_{gem}=1.0 Hz; J_{trans}=17.6 Hz); 6.68 (dd, 1H, J_{cis}=10.9 Hz; J_{trans}=17.6 Hz); 7.11 (m, 2H); 7.31 (m, 2H); 7.71 (m, 2H); 7.83 (m, 2H). ^{13}C NMR 26.72, 28.53, 28.79, 31.18, 35.54, 38.02, 112.81, 123.17, 126.13, 128.55, 132.19, 133.85, 135.10, 136.73, 142.43, 168.47. Anal. Calcd. for $C_{22}H_{23}NO_2$: C, 79.25; H, 6.95; N, 4.20. Found: C, 79.20; H, 6.93; N, 4.18.

4.5. Hydrazinolysis of Phthalimides 4a–d

Phthalimide **4a**–**d** (38.3 mmol) was dissolved in 95% ethanol (50 mL) and treated under nitrogen and stirring at reflux with a solution of hydrazine hydrate (2.74 g, 54.7 mmol) in 95% ethanol (5 mL) for 2.5 h up to the disappearance of **4a**–**d** (TLC, eluent benzene). After removal of the solvent at reduced pressure the solid residue was taken with chloroform (50 mL) and treated with 20% aqueous NaOH (50 mL). The aqueous phase was separated, extracted with chloroform (3×50 mL) and the extracts combined and dried over Na_2SO_4. The removal of chloroform afforded the free bases **5a** (90%), **5b** (92%), **5c** (80%) and **5d** (75%) as oils which were transformed into their hydrochlorides without distillation. The free base **5a** was vacuum distilled and characterized. Bp 58–60 °C/1 torr, [lit.[26]: 58–60 °C/0.7 torr]. ^1H NMR ($CDCl_3$, ppm) 1.76 (bs, 2H); 3.84 (s, 2H); 5.22 (dd, 1H, J_{gem}=0.9 Hz; J_{cis}=10.9 Hz); 5.72 (dd, 1H, J_{gem}=0.9 Hz; J_{trans}=17.6 Hz); 6.70 (dd, 1H, J_{cis}=10.9 Hz; J_{trans}=17.6 Hz); 7.24–7.39 (m, 4H).

4.6. Hydrochlorides 1a–d

A solution of the amine **5a**–**d** (25 mmol) in dry diethyl ether (500 ml) was cooled to 0 °C and treated under stirring up to saturation with dry gaseous hydrochloric acid. The white precipitate was filtered, washed with fresh ether, dried and crystallized to afford the hydrochloride derivative **1a**–**d**.

4.6.1. 4-Aminomethylstyrene hydrochloride (1a). Yield 89%. Mp 180 °C (dec.; 2-propanol); [lit.[27]: 160–170 °C (dec.)]. IR (KBr, ν, cm^{-1}) 989 and 901 (CH_2=CH). ^1H NMR (CD_3OD, ppm) 4.11 (s, 2H); 5.29 (dd, 1H, J_{gem}=0.90 Hz; J_{cis}=10.9 Hz); 5.83 (dd, 1H, J_{gem}=0.90 Hz; J_{trans}=17.6 Hz); 6.76 (dd, 1H, J_{cis}=10.9 Hz; J_{trans}=17.6 Hz); 7.42–7.52 (m, 4H). ^{13}C NMR 44.09, 115.36, 127.91, 130.35, 133.80, 137.37, 139.93. Anal. Calcd. for $C_9H_{12}ClN$: C, 63.72; H, 7.13; N, 8.26; Cl, 20.90. Found: C, 63.75; H, 7.12; N, 8.22; Cl, 20.88.

4.6.2. 4-Aminoethylstyrene hydrochloride (1b). Yield 77%. Mp 210 °C (ethanol); [lit.[16]: 210 °C]. IR (KBr, ν, cm^{-1}) 994 and 912 (CH_2=CH). ^1H NMR (CD_3OD, ppm) 2.92–3.03 (m, 2H); 3.12–3.23 (m, 2H); 5.21 (dd, 1H, J_{gem}=1.0 Hz; J_{cis}=10.9 Hz); 5.76 (dd, 1H, J_{gem}=1.0 Hz; J_{trans}=17.6 Hz); 6.72 (dd, 1H, J_{cis}=10.9 Hz; J_{trans}=17.6 Hz); 7.24–7.44 (m, 4H). ^{13}C NMR 34.22, 41.89, 114.08, 127.75, 130.03, 137.52, 137.70, 138.04. Anal. Calcd. for $C_{10}H_{14}ClN$: C, 65.39; H, 7.68; N, 7.63; Cl, 19.30. Found: C, 65.42; H, 7.69; N, 7.65; Cl, 19.30.

4.6.3. 4-Aminobutylstyrene hydrochloride (1c). Yield 80%. Mp 207–210 °C (acetonitrile); IR (KBr, ν, cm^{-1}) 985 and 905 (CH_2=CH). ^1H NMR (CD_3OD, ppm) 1.63–1.75 (m, 4H); 2.57–2.69 (m, 2H); 2.90–2.95 (m, 2H); 5.16 (dd, 1H, J_{gem}=1.1 Hz; J_{cis}=10.9 Hz); 5.71 (dd, 1H, J_{gem}=1.1 Hz; J_{trans}=17.6 Hz); 6.69 (dd, 1H, J_{cis}=10.9 Hz; J_{trans}=17.6 Hz); 7.10–7.36 (m, 4H). ^{13}C NMR 28.08, 29.16, 35.89, 40.70, 113.26, 117.32, 129.70, 136.97, 137.99, 142.63. Anal. Calcd. for $C_{12}H_{18}ClN$: C, 68.07; H, 8.57; N, 6.62; Cl, 16.74. Found: C, 68.05; H, 8.58; N, 6.64; Cl, 16.78.

4.6.4. 4-Aminohexylstyrene hydrochloride (1d). Yield 80%. Mp 150–153 °C (acetonitrile); IR (KBr, ν, cm^{-1}) 990 and 900 (CH_2=CH). ^1H NMR (CD_3OD, ppm) 1.30–1.50 (m, 4H); 1.55–1.75 (m, 4H); 2.61 (t, 2H, J=7.4 Hz); 2.90 (t, 2H, J=7.4 Hz); 5.15 (dd, 1H, J_{gem}=1.1 Hz; J_{cis}=10.9 Hz); 5.70 (dd, 1H, J_{gem}=1.1 Hz; J_{trans}=17.6 Hz); 6.69 (dd, 1H, J_{cis}=10.9 Hz; J_{trans}=17.6 Hz); 7.12–7.33 (m, 4H). ^{13}C NMR 27.31, 28.53, 29.69, 32.27, 36.42, 40.79, 113.05, 117.20, 129.63, 136.69, 138.08, 143.53. Anal. Calcd. for $C_{14}H_{22}ClN$: C, 70.13; H, 9.25; N, 5.84; Cl, 14.78. Found: C, 70.09; H, 9.26; N, 5.82; Cl, 14.81.

4.7. 2-Methoxy-6-hydroxybenzylamine hydrochloride (10)

A solution of 2-methoxy-6-methoxymethoxybenzaldehyde (**7**)[19] (1.1425 g; 5.8 mmol) in 95% ethanol (12 mL) was treated with a solution of hydroxylamine hydrochloride (0.4864 g, 7.0 mmol) in dry pyridine (2.3 mL) under stirring at room temperature for 90 min and at 0 °C for 30 min to facilitate the oxime precipitation. The white solid was filtered, dried, weighed (0.7210 g) and used without further purification. The mother liquors were concentrated to afford additional 0.2208 g of 2-methoxy-6-methoxymethoxybenzaldoxime (**8**) for an overall yield of 77%. Mp 131–135 °C; IR (ν, cm^{-1}) 3178, 1626, 1596, 1582, 1478, 1071.

A solution of **8** (3.13 g; 14.8 mmol) in 95% ethanol (43 mL) was treated with an equal volume of 2 M NaOH followed by

Raney nickel alloy (4.67 g) under stirring at room temperature for 90 min. The Raney nickel alloy was removed by filtration and washed with fresh ethanol. Filtrate and washings were combined, acidified with 0.8 M HCl (230 ml) and extracted with CH_2Cl_2 (30 mL). The aqueous phase was treated with solid KOH up to pH=14 and extracted with diethyl ether (3×30 mL). The extracts after drying over anhydrous Na_2SO_4 and removal of the solvent afforded 2-methoxy-6-methoxymethoxybenzylamine (**9**) (2.64 g, 90%). Bp 70 °C/0.02 torr.. Purity 98% by GC-FID. IR (ν, cm^{-1}) 3378, 3310, 1596, 1474, 1069. ^1H NMR ($CDCl_3$ ppm) 1.62 (bs, 2H); 3.48 (s, 3H); 3.82 (s, 3H); 3.89 (s, 2H); 5.20 (s, 2H); 6.58 (d, 1H, J_o=8.0 Hz); 6.73 (d, 1H, J_o=8.0 Hz); 7.14 (t, 1H, J_o=8.0 Hz).

A mixture of **9** (2.93 g, 14.9 mmol), methanol (150 mL) and hydrochloric acid (5 mL) was heated at 62 °C under stirring for 20 min up to the disappearance of **9** (TLC, eluent benzene/ethyl acetate=90/10). After removal of the solvent at reduced pressure the solid residue was dissolved in the minimum amount of DMF and precipitated in chloroform to afford **10** in the form of pearly flakes (2.03 g). The mother liquor after concentration and cooling afforded additional 0.39 g of **10** for an overall yield of 86%. Mp 211–214 °C. IR (ν, cm^{-1}) 3197, 3026, 1602, 1573, 1503, 1473, 1120. ^1H NMR (CD_3OD, ppm) 3.86 (s, 3H); 4.16 (s, 2H); 4.88 (s, 4H); 6.54 (m, 2H); 7.19 (m, 1H). ^{13}C NMR 33.66, 56.30, 103.12, 108.60, 109.21, 131.99, 158.21, 160.33.

4.7.1. 2-Methoxy-6-[(4-vinyl)benzyloxy]benzylamine hydrochloride (2). Sodium hydride (1.0112 g, 25.3 mmol) as a 60% dispersion in mineral oil was washed three times with pentane under nitrogen and suspended in dry DMF (84 mL). The suspension was added with a solution of **10** (2.40 g, 12.6 mmol) in dry DMF (24 mL), stirred for 90 min and treated with **3a** (1.9292 g, 12.6 mmol) under stirring at 40 °C for 23 h. The reaction mixture was hydrolyzed with 10% aqueous NaOH (40 mL) and extracted with peroxide-free diethyl ether. The extracts after drying over anhydrous Na_2SO_4 and removal of the last traces of DMF under vacuum afforded **11** as crude oil (2.3056 g) soon converted into its hydrochloride **2** as described for **1a–d**. Yield 43%. Mp 215–218 °C (acetonitrile). Purity 99% by HPLC. IR (KBr, ν, cm^{-1}) 990 and 924 (CH_2=CH). ^1H NMR (CD_3OD, ppm) 3.90 (s, 3H), 4.20 (s, 2H); 5.16 (s, 2H); 5.23 (dd, 1H, J_{gem}=1.0 Hz; J_{cis}=10.9 Hz); 5.82 (dd, 1H, J_{gem}=1.0 Hz; J_{trans}=17.6 Hz); 6.70–6.80 (m, 3H); 7.33–7.45 (m, 5H). ^{13}C NMR 33.41, 56.45, 71.50, 105.06, 106.50, 110.26, 114.46, 127.43, 128.95, 132.46, 137.70, 137.73, 138.95, 159.05, 160.28. Anal. Calcd. for $C_{17}H_{20}ClNO_2$: C, 66.77; H, 6.59; N, 4.58; Cl, 11.59. Found: C, 66.80; H, 6.58; N, 4.60; Cl, 11.62.

4.7.2. 2-Methoxy-6-[(4-vinyl)benzyloxy]benzaldehyde (12). A solution of 2-hydroxy-6-methoxybenzaldehyde[19] (0.130 g, 0.84 mmol) in dry DMF (1 mL) was treated with NaH (0.033 g, 1.10 mmol) as an 80% mineral oil dispersion at rt for 1 h. The suspension was cooled to 0 °C, **3a** (0.160 g, 1.10 mmol) was added and the mixture was heated at 40 °C for 48 h checking the progress of the reaction by TLC (benzene/ethyl acetate 70/30 as eluent). The mixture was hydrolyzed with 1 M HCl (4 mL), extracted with diethyl ether (3×8 mL) and dried over Na_2SO_4. After removal of the solvent at reduced pressure, the crude oil was purified by column chromatography (eluent chloroform) to afford 12 (0.150 g, 75%). Purity 94% by HPLC. IR (ν, cm^{-1}) 2773 (aldehydic CH), 1686 (C=O), 1596, 1475, 1109. ^1H NMR ($CDCl_3$, ppm) 3.90 (s, 3H); 5.16 (s, 2H); 5.26 (dd, 1H, J_{gem}=0.8 Hz; J_{cis}=10.8 Hz); 5.76 (dd, 1H, J_{gem}=0.8 Hz; J_{trans}=17.6 Hz); 6.61 (m, 2H); 6.72 (dd, 1H, J_{cis}=10.8 Hz; J_{trans}=17.6 Hz); 7.40–7.50 (m, 5H); 10.59 (s, 1H). GC-MS (EI, *m/z*, %): 268 (M^+, 4), 117 (100).

4.8. General procedure for solution polymerizations of 1a–d or 2 and copolymerizations with DMAA

Degassed monomers, solvent and initiator were introduced in the desired ratios under nitrogen in the polymerization flask and magnetically stirred. After a suitable period the mixture was poured into diethyl ether and the polymer was filtered, submitted to two dissolution/precipitation cycles with methanol/diethyl ether and vacuum-dried at room temperature.

4.9. General procedure for reverse-phase suspension copolymerizations

A mixture of hexane and CCl_4 was placed in a round-bottom cylindrical flanged reactor equipped with an anchor-type mechanical stirrer and nitrogen inlet, thermostated at 35 °C and deoxygenated by nitrogen bubbling for 30 min. A solution obtained by dissolving under nitrogen the monomer **1a–d** or **2**, DMAA, EBA, and APS in deoxygenated water distilled over $KMnO_4$, was siphoned into the reaction vessel. The density of the organic phase was adjusted by addition of CCl_4 so that the aqueous phase sank slowly when the stirring was stopped. The polymerization was started by setting the mechanical stirring at 900 rpm, introducing SPAN 85 and after 10 min TMEDA, and continuing the polymerization for 90 min. The resin was filtered, washed with 2-propanol, chloroform, water, absolute ethanol, chloroform, 2-propanol and acetone in order, then dried at reduced pressure and room temperature for 16–20 h and sieved. Table 1 collects data of some representative experiments.

Acknowledgements

This work was financially supported by Italian MIUR (Progetti di Rilevante Interesse Nazionale 2002–2004) and University Funds.

References and notes

1. Stubbe, J.; van der Donk, W. A. *Chem Rev.* **1998**, *98*, 705–762.
2. (a) Parsons, M. R.; Convery, M. A.; Wilmot, C. M.; Yadav, K. D. S.; Blakeley, V.; Corner, A. S.; Phillips, S. E. V.; McPherson, M. J.; Knowles, P. F. *Structure* **1995**, *3*, 1171–1184. (b) Kumar, V.; Dooley, D. M.; Freeman, H. C.; Guss, J. M.; Matsunami, H.; Harvey, I.; McGuirl, M. A.; Wilce, M. C.; Zubak, V. M. *Structure* **1996**, *4*, 943–955. (c) Wilce, M. C.; Dooley, D. M.; Freeman, H. C.; Guss, J. M.; Matsunami, H.; McIntire, W. S.; Ruggiero, C. E.; Tanizawa,

K.; Yamaguchi, H. *Biochemistry* **1997**, *36*, 16116–16133. (d) Wilmot, C. M.; Murray, J. M.; Alton, G.; Parsons, M. R.; Convery, M. A.; Blakeley, V.; Corner, A. S.; Palcic, M. M.; Knowles, P. F.; McPherson, M. J.; Phillips, S. E. V. *Biochemistry* **1997**, *36*, 1608–1620. (e) Li, R.; Klinman, J. P.; Matthews, F. S. *Structure* **1998**, *6*, 293–307. (f) Murray, J. M.; Saysell, C. G.; Wilmot, C. M.; Tambyrajah, W. S.; Jaeger, J.; Knowles, P. F.; Phillips, S. E. V.; McPherson, M. J. *Biochemistry* **1999**, *38*, 8217–8227. (g) Duff, A. P.; Cohen, A. E.; Ellis, P. J.; Kuchar, J. A.; Langley, D. B.; Shepard, E. M.; Dooley, D. M.; Freeman, H. C.; Guss, M. *Biochemistry* **2003**, *42*, 15148–15157.

3. (a) Klinman, J. P. *Biochim. Biophys. Acta* **2003**, *1647*, 131–137. (b) Mure, M. *Acc. Chem. Res.* **2004**, *37*, 131–139.
4. Mure, M.; Mills, S. A.; Klinman, J. P. *Biochemistry* **2002**, *41*, 9269–9278.
5. Wilflingseder, D.; Schwelberger, H. G. *J. Chrom. B* **2000**, *737*, 161–166.
6. Houen, G.; Jørgensen, J.; Leonardsen, L.; Larsson, L. *Acta Chem. Scand.* **1993**, *47*, 902–909.
7. Janes, S. M.; Klinman, J. P. *Biochemistry* **1991**, *30*, 4599–4605.
8. Mondovì, B.; Turini, P.; Befani, O.; Sabatini, S. *Methods Enzymol.* **1983**, *94*, 314–318.
9. Bertini, V.; De Munno, A.; Lucchesini, F.; Buffoni, F.; Bertocci, B. Ital. Patent Appl. 47906-A/85 extended to Europe, USA, Canada and Japan; *Chem. Abstr.* **1987**, *106*, 156038m.
10. Houen, G. *J. Biochem. Biophys. Methods* **2001**, *49*, 189–197.
11. Arshady, R. *J. Chrom. A* **1991**, *586*, 199–219.
12. Tamura, M.; Kochi, J. *Synthesis* **1971**, 303–305.
13. Friedman, L.; Shani, A. *J. Am. Chem. Soc.* **1974**, *96*, 7101–7103.
14. Tomoi, M.; Ogawa, E.; Hosokawa, Y.; Kakiuchi, H. *J. Polym. Sci. Chem. Ed.* **1982**, *20*, 3015–3019.
15. Andringa, H.; Hanekamp, J.; Brandsma, L. *Synth. Commun.* **1990**, *20*, 2349–2351.
16. Wulff, G.; Sarhan, A.; Gimpel, J.; Lohmar, E. *Chem. Ber.* **1974**, *107*, 3364–3376.
17. Kaufman, T. S.; Srivastava, R. P.; Sindelar, R. D.; Scesney, S. M.; Marsh, H. C., Jr. *J. Med. Chem.* **1995**, *38*, 1437–1445.
18. Pocci, M.; Bertini, V.; Lucchesini, F.; De Munno, A.; Picci, N.; Iemma, F.; Alfei, S. *Tetrahedron Lett.* **2001**, *42*, 1351–1354.
19. Narasimhan, N. S.; Mali, R. S.; Barve, M. V. *Synthesis* **1979**, 906–909.
20. Arshady, R.; Atherton, E.; Clive, D. L. J.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1* **1981**, 529–537.
21. Stahl, G. L.; Walter, R.; Smith, C. W. *J. Am. Chem. Soc.* **1979**, *101*, 5383–5394.
22. Gaur, R. K.; Gupta, K. C. *Anal. Biochem.* **1989**, *180*, 253–258.
23. Bertini, V.; Pocci, M.; Picci, N.; De Munno, A.; Lucchesini, F.; Iemma, F. *J. Polym. Sci.* **1999**, *37*, 3109–3118.
24. Hirao, A.; Hayashi, M.; Nakahama, S. *Macromolecules* **1996**, *29*, 3353–3358.
25. Kobayashi, K.; Sumimoto, H.; Ina, Y. *Polym. J.* **1983**, *15*, 667–671.
26. Suzuki, K.; Hirao, A.; Nakahama, S. *Makromol. Chem.* **1989**, *190*, 2893–2901.
27. Wulff, G.; Gimpel, J.; Feld, J.; Hufnagel, I. *Makromol. Chem.* **1982**, *183*, 2459–2467.