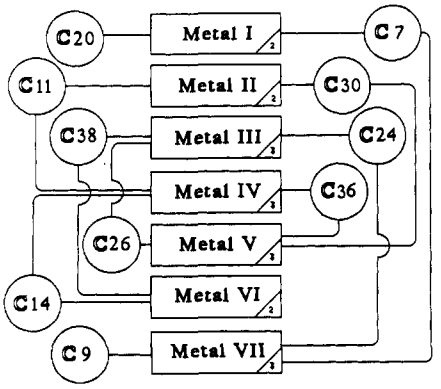


Figure 5. A schematic representation of the metal-to-cysteine connectivities. Metals (Ag(I)) are numbered by their increasing $^{109}$Ag chemical shift and are denoted by Roman numerals (Figure 3). Cysteines are labeled by their primary sequence numbers, and in the right lower corner of each rectangle, the coordination number of each Ag(I) ion is indicated.

dependence of the magnitude of the multiple quantum coherence in the HMQC experiment on the preparation period, $(1/2J)$, nevertheless, cautions against excluding the possibility that the two coordinate Ag(I) sites identified in Figure 5 may be missing their connectivities to a third cysteine due to a very small (<5 Hz) heteronuclear coupling constant. This uncertainty is unfortunately not resolved by the near-equivalence in the $H_\beta$ proton chemical shifts for four cysteines (C9, C11, C24, C38), Table I, which allowed for the unequivocal identification of only three (C11, C24, C38) as bridging cysteines on the basis of observed connectivities to both of their $H_\beta$ protons. Thus, to account for the large Ag(I) chemical shift dispersion (460 ppm) in Ag-MT, our present model invokes a mixed Ag(I) coordination number (2 and 3) involving 10 of the 12 cysteine thiols in this protein, with eight participating as bridging ligands and two as terminal ligands.

The observation of only seven Ag(I) resonances for the reconstituted Ag-MT protein is at odds with the reported metal-binding stoichiometry of eight for the Ag(I)- or native Cu(I)-MT.[7,8] It is unlikely that this results from the fact that slightly less (7.5 mol equiv) than the reported 8 mol equiv of required metal ion was intentionally added to the apoprotein to purposely avoid the possible aggregation which excess metal ions have been shown to initiate in the mammalian forms of this protein.[37] With the reported cooperative nature of metal ion binding,[8] 93% of the sample would still contain its full complement of eight metal ions. Other possible explanations such as degeneracy of two Ag(I) resonances and limitations in the size of the $^{109}$Ag(I) spectral window, which covered the range of 545 to 1551 ppm, seem equally unlikely. The possibility remains, nevertheless, that the eighth resonance is exchange-broadened, and one is reminded of the fact that no cross-peaks were observed in the HMQC data sets to the $H_\beta$ protons from the two C-terminal cysteine residues (C49 and C50).

Our current model, therefore, suggests that the factors affecting the chemical shifts of the seven Ag(I) thiolate resonances in Ag-MT includes both (a) coordination number of the metal ions[27,32,33] and (b) the bond distances, bond angle, and number of bridging versus terminal thiolates.[34] These metal-to-cysteine connectivities (Figure 5) will provide crucial constraints for the determination of the three-dimensional solution structure of Ag-MT by NMR methods that is currently in progress. Also planned are HMQC experiments and the full 3D solution structural NMR studies on the mutant MT which lacks the five C-terminal residues, which include two cysteines. Together, these studies should provide unequivocal determination of the Ag(I) and, therefore, presumably the Cu(I) metal ion stoichiometry and coordination number in MT from *Saccharomyces cerevisiae*.

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**Registry No.** cysteine, 52-90-4; copper, 7440-50-8.

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# *Communications to the Editor*

## Direct Enantioseparation of β-Adrenergic Blockers Using a Chiral Stationary Phase Prepared by Molecular Imprinting

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β-Adrenergic blocking agents, or β-blockers, are important drugs used for the treatment of hypertension, arrhythmia, and angina pectoris. There is a strong need to apply optically pure enantiomers since the stereoisomers express varying pharmacological activity and, in some cases, can even be used against different symptoms.[1] Thus, there is intense ongoing research in the preparation of optically pure β-blockers such as the use of asymmetrical synthesis[2] (including biocatalysts[3]), fractionated crystallization,[4] and indirect[5] or direct[6] chromatographic separation of the enantiomeric forms. In this communication we describe,

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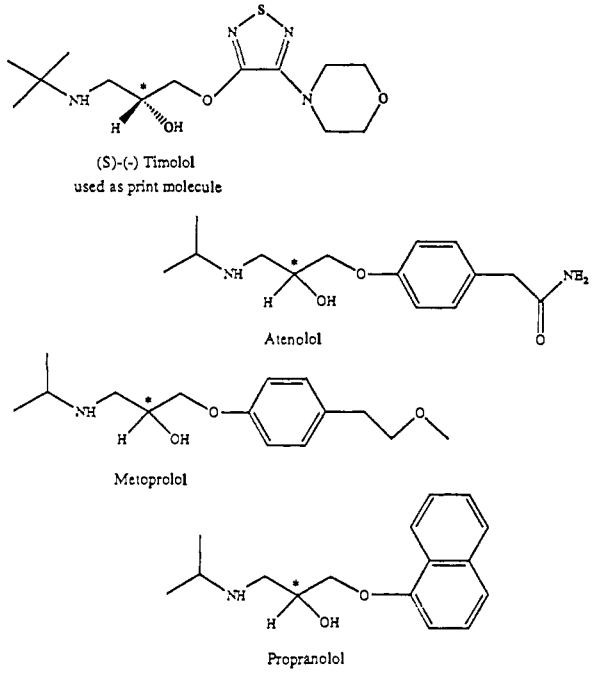


**Figure 1.** Structures of the various β-blockers tested. The *S*-(−) configuration of timolol was used as the print molecule for the preparation of the chiral stationary phases (CSP).

to the best of our knowledge, the first direct chiral separation of β-blockers using as a chiral stationary phase (CSP) column material prepared by the technique of molecular imprinting.[7] The molecular imprinting technique used here is based on noncovalent complementary interactions between the nonderivatized print molecules (template) and polymerizable monomers.

Two different monomers, methacrylic acid [MAA, $H_2C{=}C(CH_3)COOH$] and itaconic acid [ITA, $H_2C{=}C(COOH)CH_2COOH$], were tested. The use of ITA is well established in polymer chemistry.[8] However, we describe here for the first time its use as a monomer for molecular imprinting.

Both monomers have the property of forming, through their functional groups, noncovalent linkages in organic solvents with the print molecule, such as (*S*)-(−)-timolol (Figure 1). The presence of a cross-linking agent, such as ethylene glycol dimethacrylate, during polymerization results in the formation of a three-dimensional network. The print molecule is subsequently removed from this network by extraction with 20% acetic acid in acetonitrile. This is likely to leave individual sites with complementary binding points within the polymer.[7d]

Both CSPs obtained using imprints of (*S*)-(−)-timolol allowed base-line separation on subsequent application of a racemic mixture of timolol with $R_S$ values between 1.9 and 2.0 (see Table I and Figure 2A,B). The CSP obtained with methacrylic acid (MAA-CSP) also allowed separation into enantiomeric forms of other β-blockers applied (see Figure 1). However, in this context racemic mixtures of metoprolol and atenolol were resolved poorly (data not shown), but propranolol was resolved satisfactorily ($k'_R$ = 1.0; $\alpha$ = 2.8; $R_S$ 1.3). This is in accordance with previous findings of the enantiomeric separation of amino acid derivatives of structurally related molecules with MAA-polymers.[9] Due to its high optical rotation values, the enantioseparation of propanolol could also be determined polarimetrically when the sample concentration was increased to 20 g/L. It was thus established that the peaks obtained from the separated enantiomers were identical to those measured by UV absorption.

In the case of itaconic acid based imprinted polymers (ITA-CSP), not only were sharper peaks obtained (Figure 2B) but also a higher degree of selectivity was displayed. Using (*S*)-(−)-timolol as print molecule and applying subsequently an artificial mixture of the racemic (aryloxy)propanolamines timolol, propranolol, atenolol, and metoprolol (for structures, see Figure 1), (*S*)-(−) timolol was by far most efficiently retarded whereas all the others were not separated into their enantiomeric compounds nor did they bind to any substantial degree (propanolol, $k'_{rac}$ = 0.2; atenolol and metoprolol, $k'_{rac}$ = 2.2; timolol, $k'_R$ = 2.5 and $k'_S$ = 3.6; flow rate, 0.4 mL/min; UV detection, 275 nm). These results are possibly due to the adjacent carboxylic groups of the bifunctional monomer itaconic acid used and its more pronounced possibility of interactions with the heterocyclic side chain of timolol.

We feel that the results obtained, the resolution of racemic mixtures of important nonderivatized pharmaceuticals like β-blockers with the molecular imprinting technique, bears in itself great potential. In this context it deserves mentioning that the separation properties persisted during a period of 8 months and more than 50 injections. In contrast to other methods applied until now, it offers a large degree of freedom in that it allows the creation of specific separation materials with predictable selectivity

**Table I.** Chromatographic Resolution of Timolol on Chiral Stationary Phases[a] (CSPs) Prepared by the Molecular Imprinting Techinque Using (*S*)-(−)-Timolol as the Print Molecule

| functional monomer in the chiral stationary phase | $k'_R$ | $\alpha$ | $R_S$ |
|---|---|---|---|
| methacrylic acid (MAA)[b] | 2.0 | 2.9 | 2.0 |
| itaconic acid (ITA)[b] | 1.4 | 2.5 | 1.9 |

[a] Conditions and eluents are given in Figure 2. [b] The composition of the polymers was 2 mmol of (*S*)-(−)-timolol, 12 mmol of monomer MAA (or ITA), and 60 mmol of ethylene glycol dimethacrylate in 20 mL of chloroform (or tetrahydrofuran). The polymerization was initiated by photolytic homolysis of azobis(nitriles) (0.5 mmol) at 366 nm.[10] The bulk polymers were ground and the resulting particles sieved, sedimented, and packed in HPLC columns as described elsewhere[11] [particle size, 25 μm; packing conditions, 300 bar; column size, 200 × 4.6 (i.d.) mm]. The retention time $t_0$ for nonretarded solutes was determined by the injection of acetone; $k'_R = (t_R - t_0)/t_0$; $k'_S = (t_S - T_0)/t_0$; $\alpha = k'_S/k'_R$; $R_S$ was determined graphically;[7] $k'_R$ is the capacity factor of the *R*-(+) enantiomer; $k'_S$ is the capacity factor of the *S*-(−) enantiomer; and $R_S$ is the resolution.

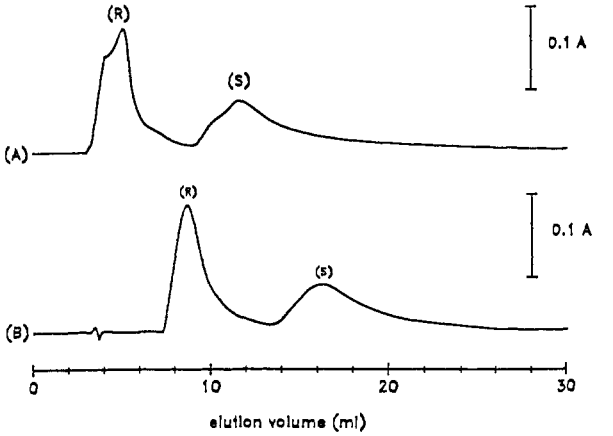


**Figure 2.** Chromatographic resolution of timolol on polymers containing (A) methacrylic acid and (B) itaconic acid. The optimized eluents chosen were acetonitrile/acetic acid (93/7 v/v) for A and ethanol/tetrahydrofuran/acetic acid (50/40/10 v/v/v) for B. The sample volume was 20 μL containing 20 μg of β-blocker, the flow rate was 1 mL/min, and the pressure was approximately 30 bar. All separations were carried out at ambient temperature, and UV detection was monitored at 294 nm. The elution order was established by injection of the pure enantiomers.

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at will. Although the capacity shown here compares more than well with, for example, a biological alternative method using the protein cellulase[6d] [the amounts of timolol with optimal base-line separation were 18.9 and 19.9 μg/g of dry CSP, respectively (Figure 2), and in connection with the aforementioned polarimetric study of propranolol, with fair resolution but no base-line separation, 400 μg/g of dry CSP], work aimed at increasing the capacity of analogous imprinted polymers is in progress and will further increase the applicability of the technique for (semi)-preparative isolation of pure enantiomers of compounds such as β-blockers. However, we feel that already today the removal of contaminating small amounts of the nondesired enantiomer may be feasible on a technical scale with the described technique.

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## Lipase-Catalyzed Kinetic Resolution with in Situ Racemization: One-Pot Synthesis of Optically Active Cyanohydrin Acetates from Aldehydes

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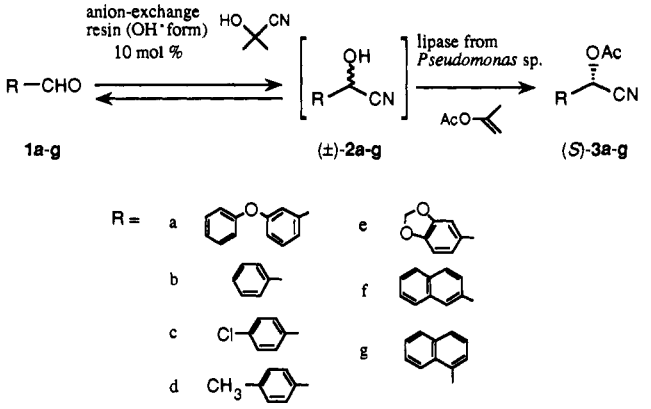
Lipase-catalyzed kinetic resolution of racemic compounds has been recognized as an effective tool for the preparation of optically active compounds.[1] Like conventional resolution processes, however, the maximum yield of one enantiomer cannot exceed 50%. Therefore, the development of a method which includes in situ racemization of the substrate is a challenging objective, because it would allow for the quantitative conversion of the racemic substrate into a single enantiomer. This type of enzymatic second-order asymmetric transformation has only been achieved in a limited number of cases.[2,3]

Cyanohydrins are promising candidates for such a second-order asymmetric transformation, because they can be reversibly decomposed into achiral aldehydes under basic conditions; moreover, they are easily converted into various useful chiral synthons. Although numerous examples of the enzyme-catalyzed synthesis of optically active cyanohydrins have been reported,[4] many of them have been based on simple kinetic resolution, and, in some cases, one of the enantiomers was decomposed or racemized.[4a,b,d-g]

**Scheme I**




We now report a novel one-pot synthesis of optically active cyanohydrin acetates from aldehydes, through the combination of in situ cyanohydrin formation with a lipase-catalyzed kinetic resolution of cyanohydrins in an organic solvent (Scheme I). Aromatic aldehydes **1a-g** were reversibly converted into the corresponding cyanohydrins **2a-g** through transhydrocyanation with acetone cyanohydrin, catalyzed by an anion-exchange resin ($OH^-$ form). The resulting cyanohydrins were enantioselectively acetylated by subsequent lipase-catalyzed transesterification using isopropenyl acetate as an acyl donor[5] to yield optically active cyanohydrin acetates **3a-g** having high enantiomeric excesses. Due to the reversible nature of the base-catalyzed transhydrocyanation,[6] cyanohydrin **2** is subject to rapid racemization. When the acidic hydroxy group in **2** is protected by acetylation,[7] racemization is greatly reduced and hence cyanohydrins **2** formed in situ from aldehydes **1** are converted into a single stereoisomer of the cyanohydrin acetate **3**. Acetone cyanohydrin, which is easier to handle than HCN, was used as the hydrogen cyanide source for this reaction.[8] It produces acetone as the only byproduct after the release of HCN. Importantly, acetone cyanohydrin is not subject to acetylation by lipase probably due to its steric bulk.[9] Therefore, it remains an effective HCN donor throughout the reaction.

The results are summarized in Table I. A lipase from *Pseudomonas* sp. M-12-33 (Amano)[10] and three types of strongly basic, macroporous anion-exchange resins ($OH^-$ form, 10 mol % equiv) were used as catalysts. The reaction was monitored by $^1H$ NMR, and the degree of cyanohydrin formation and enzymatic acetylation were calculated from the ratio of each component **1**, **2**, and **3** found in the reaction mixture. 3-Phenoxybenzaldehyde (**1a**) was transformed into the corresponding acetate **3a** in 3 days via a 95% conversion in the transhydrocyanation (from **1a** to **2a**) and an 88% conversion in the enzymatic acetylation (from **2a** to **3a**).

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(9) Acetone cyanohydrin was not acetylated by the lipase (from *Pseudomonas* sp.) after incubation (40 °C, 6.7 days) with isopropenyl acetate in diisopropyl ether.

(10) The lipase from *Pseudomonas* sp. M-12-33 (Amano Pharm. Co., Ltd.) was obtained as lyophilized powder and used after immobilization as follows: the lipase powder was dissolved in 20 mM Tris-HCl buffer (pH 8.0) and mixed with Hyflo Super-Cel (10 mg lipase/g Hyflo Super-Cel). The resulting paste was dried in a desiccator over $P_2O_5$ (Inagaki, M.; Hiratake, J.; Nishioka, T.; Oda, J. *Agric. Biol. Chem.* **1989**, *53*, 1879-1884).

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