# Molecular Imprinting Utilizing an Amide Functional Group for Hydrogen Bonding Leading to Highly Efficient Polymers

Cong Yu and Klaus Mosbach*

*Department of Pure and Applied Biochemistry, Chemical Center, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden*

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In this paper we report the synthesis of molecularly imprinted polymers (MIPs) using an amide as the hydrogen-bonding functional group. All the amide MIPs made in this investigation exhibited good enantiomeric recognition of the imprinted species when evaluated by high-performance liquid chromatography (HPLC). Most of the amide MIPs were synthesized in a polar organic solvent (acetonitrile). By contrast, when a carboxyl group was used as the hydrogen-bonding functional group, MIPs made in acetonitrile exhibited only very weak recognition and in some cases no recognition at all with the print molecules tested. The amide MIPs also showed much improved load capacity. This suggests that for a number of cases the protocol described could be employed to reduce background, nonspecific binding compared to polymers using carboxyl groups, by avoiding an excess of negatively charged functionalities. It was shown that the tailing and peak asymmetry problems often associated with the use of MIPs in HPLC could be overcome by using linear gradient elution. The specificities of amide MIPs in terms of size, shape, and hydrogen-bonding selectivity were also investigated in detail. Finally, amide MIPs also showed good enantiomeric recognition in aqueous media.

## Introduction

The fabrication of artificial receptors that can achieve recognition at the molecular level is one of the major goals of organic and bioorganic chemistry. On the basis of the increasing understanding of the basic interactions (hydrogen bonding, ionic interactions, van der Waals interactions, the hydrophobic effect, metal chelation, etc.) between molecules and of the recognition between substrate−enzyme, antigen−antibody, and ligand−receptor, several well-known synthetic recognition systems have been developed,[1] and newly synthesized receptors are emerging very rapidly.[2]

Molecular imprinting is a rapidly developing technique for the preparation of such artificial receptors and has recently been extensively reviewed.[3−6] Generally, when a template, a functional monomer, and a cross-linker are mixed together in an organic solvent, a complex is formed between the template and the functional monomer through polar interactions. Subsequent polymerization with the cross-linker fixes the positions of the polar groups of the functional monomer. Finally, washing the template away leaves recognition sites that are specific for the template molecules. Materials prepared by molecular imprinting have been used successfully for chiral separations of amino acid derivatives,[7] drugs,[8] and sugar derivatives,[9] for specific recognition of steroids,[10] proteins, and protein analogues,[11] as antibody and receptor mimics,[12] as ion selective absorbents,[13] and as enzyme mimics to direct organic reactions.[14−17]

The normal procedure in the preparation of synthetic imprinted polymers involves, apart from the cross-linker (ethylene glycol dimethacrylate), charged functional monomers, mostly methacrylic acid. As with the latter approach, excess of carboxylic groups often remain on the polymer. We investigated whether acrylamide could be used instead. In addition, as hydrogen-bonding interactions play a crucial role in biological recognition systems and in determining the structures of proteins and nucleic

* Tel: +46-46-2228260. Fax: +46-46-2224611. E-mail: Klaus.Mosbach@tbiokem.lth.se.

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(1) (a) Cram, D. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 1009. (b) Pedersen, C. J. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 1021. (c) Lehn, J. M. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 89. (d) Rebek, J. *Acc. Chem. Res.* **1990**, *23*, 399. (e) Schneider, H. J. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1417. (f) Wenz, G. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 803.

(2) (a) Kobuke, Y.; Kokubo, K.; Munakata, M. *J. Am. Chem. Soc.* **1995**, *117*, 12751. (b) Granados, A.; de Rossi, R. H. *J. Am. Chem. Soc.* **1995**, *117*, 3690. (c) Kuroda, Y.; Kato, Y.; Higashioji, T.; Hasegawa, J.-Y.; Kawanami, S.; Takahashi, M.; Shiraishi, N.; Tanabe, K.; Ogoshi, H. *J. Am. Chem. Soc.* **1995**, *117*, 10950. (d) Bonar-Law, R. P.; Sanders, J. K. M. *J. Am. Chem. Soc.* **1995**, *117*, 259. (e) Famulok, M. *J. Am. Chem. Soc.* **1994**, *116*, 1698.

(3) (a) Mosbach, K. *Trends Biol. Sci.* **1994**, *19*, 9. (b) Ansell, R. J.; Mosbach, K. *Pharmaceutical News.* **1996**, *3*, 16. (c) Mosbach, K.; Ramström, O. *Bio/technology* **1996**, *14*, 163.

(4) Wulff, G. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1812.

(5) Shea, K. J. *Trends Polym. Sci.* **1994**, *2*, 166.

(6) Vidyasankar, S.; Arnold, F. H. *Curr. Opin. Biotechnol.* **1995**, *6*, 218.

(7) (a) Sellergren, B.; Lepistö, M.; Mosbach, K. *J. Am. Chem. Soc.* **1988**, *110*, 5853. (b) Andersson, L. I.; O'Shannessy, D. J.; Mosbach, K. *J. Chromatogr.* **1990**, *513*, 167. (c) Andersson, L. I.; Mosbach, K. *J. Chromatogr.* **1990**, *516*, 313. (d) Ramström, O.; Andersson, L. I.; Mosbach, K. *J. Org. Chem.* **1993**, *58*, 7562.

(8) (a) Fischer, L.; Müller, R.; Ekberg, B.; Mosbach, K. *J. Am. Chem. Soc.* **1991**, *113*, 9358. (b) Kempe, M.; Mosbach, K. *J. Chromatogr.* **1994**, *664*, 276.

(9) (a) Wulff, G.; Haarer, J. *Makromol. Chem.* **1991**, **192**, 1329. (b) Mayes, A. G.; Andersson, L. I.; Mosbach, K. *Anal. Biochem.* **1994**, *222*, 483.

(10) Whitcombe, M. J.; Rodriguez, M. E.; Villar, P.; Vulfson, E. N. *J. Am. Chem. Soc.* **1995**, *117*, 7105.

(11) (a) Mallik, S.; Johnson, R. D.; Arnold, F. H. *J. Am. Chem. Soc.* **1994**, *116*, 8902. (b) Kempe, M.; Glad, M.; Mosbach, K. *J. Mol. Recogn.* **1995**, *8*, 35.

(12) (a) Vlatakis, G.; Andersson, L. I.; Müller, R.; Mosbach, K. *Nature* **1993**, *361*, 645. (b) Andersson, L. I.; Müller, R.; Vlatakis, G.; Mosbach, K. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 4788.

(13) Rosatzin, T.; Andersson, L. I.; Simon, W.; Mosbach, K. *J. Chem. Soc., Perkin Trans. 2* **1991**, 1261.

(14) (a) Ståhl, M.; Jeppsson-Wistrand, U.; Månsson, M.-O. *J. Am. Chem. Soc.* **1991**, *113*, 9366. (b) Mingarro, I.; Abad, C.; Braco, L. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3308.

(15) Byström, S. E.; Börje, A.; Åkermark, B. *J. Am. Chem. Soc.* **1993**, *115*, 2081.

(16) Robinson, D. K.; Mosbach, K. *J. Chem. Soc., Chem. Commun.* **1989**, *14*, 969.

(17) Beach, J. V.; Shea, K. J. *J. Am. Chem. Soc.* **1994**, *116*, 379.

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acids.[18,19] We considered it would also be of interest to utilize strong hydrogen-bonding interactions to create imprinted polymers with specific recognition properties.

Many attempts have been made in recent years to determine the hydrogen-bonding patterns between molecules and their relative strengths both theoretically and experimentally.[18−28] The results indicate that hydrogen bonding with a charged group is much stronger than with a noncharged group. The strengths of hydrogen-bonding interactions are greatly modulated by the solvent. Cooperativity has also been observed: The enthalpy of a cyclic dimer containing two hydrogen bonds is more than twice that of the open dimer containing only one hydrogen bond. For amides and carboxylic acids, a number of different hydrogen-bonding complexes have been studied in the gas phase; it was found that O−H···O and N−H···O hydrogen bonds are the strongest among all kinds of hydrogen-bonding complexes containing only one hydrogen bond. For formic acid, it was shown that the gas phase structure is completely different from the liquid and solid phases.

Currently, the carboxyl group is the most commonly used hydrogen-bonding functional group in molecular imprinting. Although it can form strong ionic interactions with basic functional groups, the hydrogen-bonding ability of this functional group is not very strong in polar solvents. MIPs made in a polar solvent using carboxyl functional monomers and print molecules which could only form hydrogen bond interactions have often exhibited weak recognition, and in some cases no recognition at all.[7cd,29]

By contrast, although no general agreement about the relative strength of amide and carboxylic acid hydrogen bonds has been reached,[22−24] and our imprinting solutions are quite different from pure gas, liquid, and solid phases, several clues suggest that the amide functional group may be capable of forming strong hydrogen bonds in polar solvents.

(a) Previous results show that a polymer imprinted against a template having an amide group instead of an ester group normally gives much better enantiomeric resolution.[7ab,30,31]

(b) The large differences between dielectric constants and dipole moments of the amide group and the carboxyl group also suggest that the amide group may form stronger hydrogen bonds than the carboxyl group. Thus, acetic acid has a dielectric constant value of 6.20, and acetamide has a value of 67.6. The dipole moment of acetic acid is 1.70 D, while for acetamide this value is 3.76 D.[32]

(c) In a peptide bond, the amide oxygen has 0.42 negative charge and the hydrogen has 0.20 positive charge.[33] This also suggests that the amide group may form strong hydrogen bonds in water.

Amide monomers could also be used in combination with different functional monomers. For templates having both hydrogen bonding and acidic functional groups, the combination of methacrylic acid and a basic functional monomer (vinylpyridine) has previously been shown to give MIPs with improved enantiomeric recognition.[7d] One problem with this combination is that the ionic interaction between these two functional monomers will compete with their interactions with the print molecule and may decrease the imprinting efficiency. By using vinylpyridine with an amide monomer instead of methacrylic acid, this problem may be solved. Other combinations (such as methacrylic acid and acrylamide) could also be used for different templates. We anticipate that, using such combinations, molecularly imprinted polymers could be made with improved recognition properties.

Since biological recognition mainly occurs in aqueous systems, it is quite important to make MIPs capable of recognition in water in order to mimic biomolecules. Unlike the carboxyl group the amide group is not ionizable, which could be advantageous for molecular recognition in water.

So far, most investigations of MIPs have concentrated on enantiomeric recognition, whereas investigations concerning the various structures of ligands have not been focused on to the same degree. In our selectivity study, three kinds of amino acid derivatives (tryptophan, tyrosine, and phenylalanine) were chosen and comparisons were made of their retentions and separations by different MIPs in high-performance liquid chromatography (HPLC) in order to reveal how MIPs recognize different molecules with specificity (Figure 1).

## Results and Discussion

**Amide MIPs Showed Better Recognition Properties than Carboxyl MIPs in This Series of Experiments.** Generally speaking, MIPs prepared by polymerizing in a relatively nonpolar organic solvent exhibit better recognition properties than those prepared using a polar organic solvent. This is because the hydrogen-bonding strength is very much modulated by the medium, and the functional group of the monomer associates more weakly with the template molecules in a polar solvent. Better recognition sites are also expected using templates having more groups capable of noncovalent interactions. Thus, one common problem is that many such compounds are not very soluble in nonpolar organic solvents. Because of this, the development of a method for making efficient MIPs in polar organic solvents is of general interest.

Our results show that amide could be a promising functional group to form strong hydrogen bonds with template molecules in polar solvents. Acetonitrile and chloroform are the most commonly used solvents for

(18) Jeffrey, G. A.; Seanger, W. *Hydrogen Bonding in Biological Structures*; Springer-Verlag: Berlin, 1991.
(19) Fersht, A. R. *Trends Biol. Sci.* **1987**, *12*, 301.
(20) Alemán, C.; Navas, J. J.; Muñoz-Guerra, S. *J. Phys. Chem.* **1995**, *99*, 17653.
(21) Turi, L. *J. Phys. Chem.* **1996**, *100*, 11285.
(22) Turi, L.; Dannenberg, J. J. *J. Phys. Chem.* **1993**, *97*, 12197.
(23) Neuheuser, T.; Hess, B. A.; Reutel, C.; Weber, E. *J. Phys. Chem.* **1994**, *98*, 6459.
(24) Turi, L. *J. Phys. Chem.* **1996**, *100*, 11285.
(25) Sneddon, S. F.; Tobias, D. J.; Brooks, C. L., III *J. Mol. Biol.* **1989**, *209*, 817.
(26) Dixon, D. A.; Dobbs, K. D.; Valentini, J. J. *J. Phys. Chem.* **1994**, *98*, 13435.
(27) Alemán, C.; Vega, M. C.; Tabernero, L.; Bella, J. *J. Phys. Chem.* **1996**, *100*, 11480.
(28) Wolfs, I.; Desseyn, H. O. *Spectrochim. Acta Part A* **1995**, *51*, 1601.
(29) Kempe, M. Ph.D. Thesis, University of Lund, 1994, p 44.
(30) Andersson, L.; Sellergren, B.; Mosbach, K. *Tetrahedron Lett.* **1984**, *25*, 5211.
(31) Sellergren, B.; Ekberg, B.; Mosbach, K. *J. Chromatogr.* **1985**, *347*, 1.
(32) Lide, D. R. *CRC Handbook of Chemistry and Physics*; CRC Press: Boca Raton, 1994; pp 6-155, 9-42.
(33) Creighton, T. E. *PROTEINS Structure and Molecular Properties;* W. H. Freeman: New York, 1993; p 145.

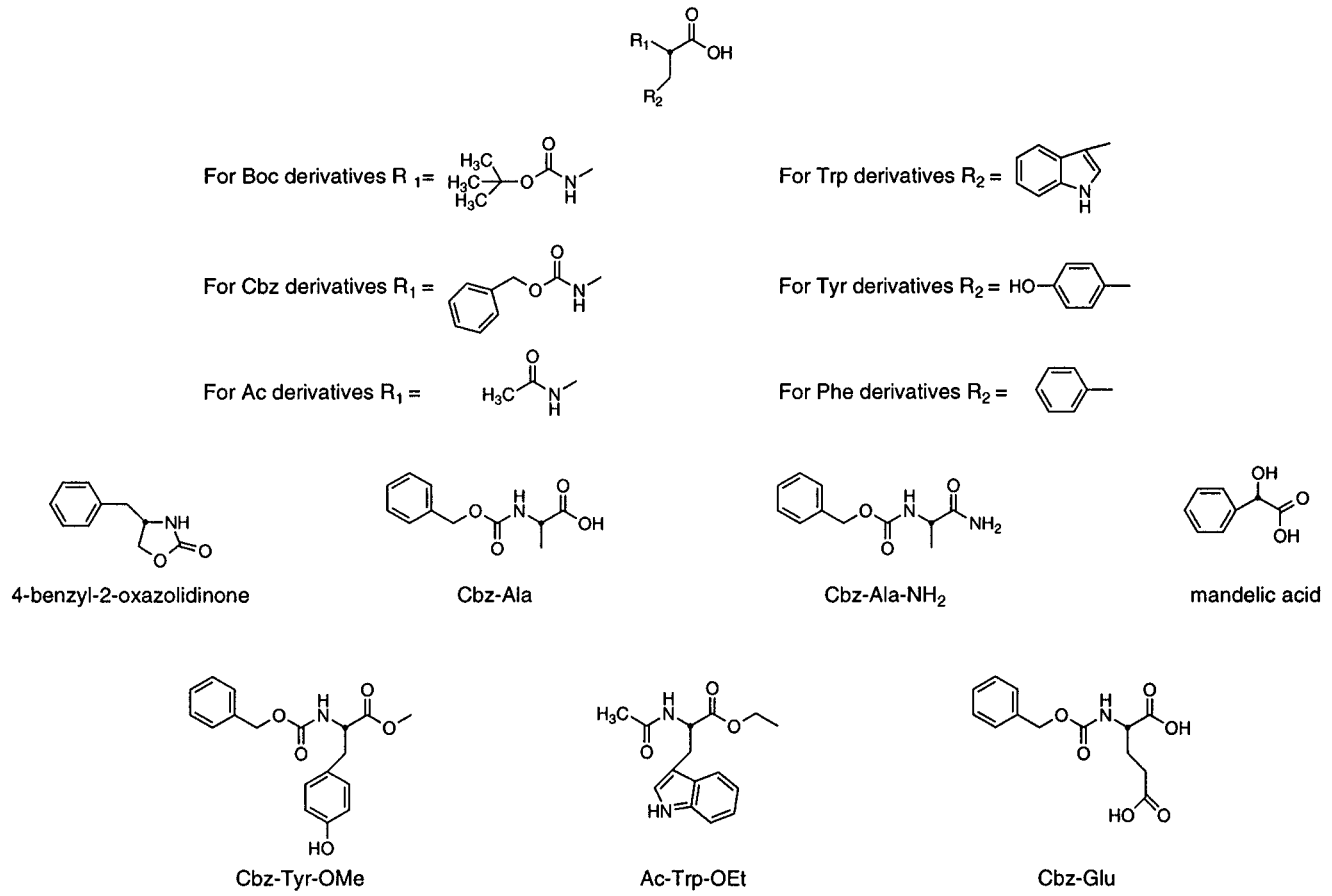



**Figure 1.** Templates and test compounds.

imprinting. Acetonitrile is much more polar than chloroform: the dielectric constant for acetonitrile is 36.64, while this value for chloroform is 4.81.[32] Previous studies have shown that when the imprint molecule is capable of ionic interactions with the carboxyl monomers, MIPs made using either solvent exhibit good recognition, but when only hydrogen bonding interactions between the imprint molecule and the carboxyl monomers are involved, MIPs made in acetonitrile exhibit only very weak enantiomeric recognition[7d] and in some cases no recognition at all.[29] When acetonitrile was used as the imprinting solvent, carboxyl MIPs imprinted against Boc-L-Trp and Cbz-L-Tyr gave only very slight enantiomeric recognition ($\alpha = 2.03$, $R_s = 0.16$; $\alpha = 1.82$, $R_s = 0.3$ respectively), and when made against Boc-L-Phe, Cbz-L-Phe, or Cbz-L-Ala, no enantiomeric recognition was observed. By contrast, most of the amide MIPs made in acetonitrile in this investigation gave good enantiomeric recognition (Tables 1−8). The results clearly show that the amide group can form much stronger hydrogen bonds with the templates in acetonitrile than the carboxyl group.

An amide MIP made against Ac-L-Trp-OEt using acetonitrile as solvent gave only weak enantiomeric separation; 0.4 $\mu$g of Ac-D,L-Trp-OEt was separated with $\alpha = 1.77$ and $R_s = 0.49$. But the amide MIP made against the same template using chloroform as solvent gave much better separation with $\alpha = 1.97$ and $R_s = 2.60$ (Table 8). This demonstrates further that MIPs made in a relative nonpolar solvent exhibit better recognition properties.

Amide MIPs also showed much improved capacities in comparison to carboxyl MIPs. For the amide MIP made against Boc-L-Trp, 40 $\mu$g of Boc-D,L-Trp could be separated with an $\alpha$ value of 3.68 and an $R_s$ value of 2.24, while a 100 $\mu$g sample could be separated with an $\alpha$ value of 2.88 and an $R_s$ value of 1.39, and a 500 $\mu$g sample could

**Table 1. Chromatographic Results for Enantiomeric Resolution of Structurally Related Compounds on an Amide MIP Prepared Using Boc-L-Trp as the Template**

| test compound | $k'_D$ | $k'_L$ | $\alpha$ | $R_s$ |
|---|---|---|---|---|
| Boc-Trp | 1.44 | 5.30 | 3.68 | 2.24 |
| Boc-Tyr | 1.51 | 2.46 | 1.63 | 0.93 |
| Boc-Phe | 0.78 | 1.23 | 1.58 | 0.81 |
| Cbz-Trp | 2.04 | 3.79 | 1.86 | 1.27 |
| Cbz-Tyr | 2.50 | 2.95 | 1.18 | 0.26 |
| Cbz-Phe | 1.23 | 1.14 | 1.15 | 0.15 |
| Ac-Trp | 1.77 | 2.41 | 1.36 | 0.56 |
| Ac-Tyr | 2.07 | 2.07 | | |
| Ac-Phe | 1.06 | 1.06 | | |
| Ac-Trp-OEt | 0.13 | 0.13 | | |
| Boc-Tyr-OMe | 0.12 | 0.12 | | |

**Table 2. Chromatographic Results for Enantiomeric Resolution of Structurally Related Compounds on an Amide MIP Prepared Using Boc-L-Tyr as the Template**

| test compound | $k'_D$ | $k'_L$ | $\alpha$ | $R_s$ |
|---|---|---|---|---|
| Boc-Trp | 2.04 | 2.86 | 1.40 | 0.94 |
| Boc-Tyr | 3.48 | 9.92 | 2.86 | 2.63 |
| Boc-Phe | 1.31 | 2.19 | 1.68 | 1.59 |
| Cbz-Trp | 3.08 | 3.54 | 1.15 | 0.28 |
| Cbz-Tyr | 5.44 | 9.46 | 1.74 | 1.46 |
| Cbz-Phe | 2.02 | 2.49 | 1.24 | 0.61 |
| Ac-Trp | 3.25 | 3.25 | | |
| Ac-Tyr | 5.06 | 6.61 | 1.31 | 0.62 |
| Ac-Phe[a] | 1.83 | 1.83 | | |
| Ac-Trp-OEt | 0.10 | 0.10 | | |
| Boc-Tyr-OMe | 0.25 | 0.25 | | |

[a] Injection of 1/10 amount of sample gave $k'_D = 1.74$, $k'_L = 2.06$, $\alpha = 1.18$, $R_s = 0.26$.

**Table 3. Chromatographic Results for Enantiomeric Resolution of Structurally Related Compounds on an Amide MIP Prepared Using Boc-L-Phe as the Template**

| test compound | $k'_D$ | $k'_L$ | α | $R_s$ |
|---|---|---|---|---|
| Boc-Trp | 1.70 | 2.33 | 1.37 | 0.73 |
| Boc-Tyr | 2.44 | 3.93 | 1.62 | 1.14 |
| Boc-Phe | 1.18 | 2.38 | 2.03 | 1.73 |
| Cbz-Trp | 2.63 | 2.90 | 1.11 | 0.12 |
| Cbz-Tyr | 3.77 | 4.99 | 1.19 | 0.35 |
| Cbz-Phe | 1.81 | 2.39 | 1.33 | 0.69 |
| Ac-Trp | 2.59 | 2.59 | | |
| Ac-Tyr | 3.90 | 3.90 | | |
| Ac-Phe | 1.55 | 1.69 | 1.10 | 0.14 |
| Ac-Trp-OEt | 0.16 | 0.16 | | |
| Boc-Tyr-OMe | 0.19 | 0.19 | | |

**Table 4. Chromatographic Results for Enantiomeric Resolution of Structurally Related Compounds on an Amide MIP Prepared Using Cbz-L-Trp as the Template**

| test compound | $k'_D$ | $k'_L$ | α | $R_s$ |
|---|---|---|---|---|
| Boc-Trp | 1.08 | 1.83 | 1.69 | 0.65 |
| Boc-Tyr[a] | 1.22 | 1.22 | | |
| Boc-Phe[b] | 0.75 | 0.75 | | |
| Cbz-Trp | 2.06 | 8.23 | 4.00 | 1.88 |
| Cbz-Tyr | 2.02 | 3.60 | 1.78 | 0.77 |
| Cbz-Phe | 1.19 | 1.98 | 1.66 | 0.74 |
| Ac-Trp | 1.50 | 2.61 | 1.74 | 0.72 |
| Ac-Tyr[c] | 1.77 | 1.77 | | |
| Ac-Phe[d] | 0.99 | 0.99 | | |
| Ac-Trp-OEt | 0.21 | 0.21 | | |
| Boc-Tyr-OMe | 0.16 | 0.16 | | |

[a] Injection of 1/10 amount of sample gave $k'_D = 1.34$, $k'_L = 1.83$, $\alpha = 1.37$, $R_s = 0.14$. [b] Injection of 1/10 amount of sample gave $k'_D = 0.73$, $k'_L = 0.92$, $\alpha = 1.26$, $R_s = 0.10$. [c] Injection of 1/10 amount of sample gave $k'_D = 2.09$, $k'_L = 2.72$, $\alpha = 1.30$, $R_s = 0.11$. [d] Injection of 1/10 amount of sample gave $k'_D = 1.23$, $k'_L = 1.50$, $\alpha = 1.22$, $R_s = 0.08$.

**Table 5. Chromatographic Results for Enantiomeric Resolution of Structurally Related Compounds on an Amide MIP Prepared Using Cbz-L-Tyr as the Template**

| test compound | $k'_D$ | $k'_L$ | α | $R_s$ |
|---|---|---|---|---|
| Boc-Trp | 2.28 | 2.56 | 1.13 | 0.08 |
| Boc-Tyr | 3.54 | 5.68 | 1.60 | 1.15 |
| Boc-Phe | 1.25 | 1.50 | 1.20 | 0.33 |
| Cbz-Trp | 3.53 | 5.26 | 1.50 | 0.96 |
| Cbz-Tyr | 6.80 | 24.6 | 3.62 | 2.52 |
| Cbz-Phe | 2.28 | 4.32 | 1.90 | 1.68 |
| Ac-Trp[a] | 3.55 | 3.55 | | |
| Ac-Tyr | 5.72 | 8.93 | 1.56 | 1.08 |
| Ac-Phe | 1.93 | 2.24 | 1.16 | 0.31 |
| Ac-Trp-OEt | 0.19 | 0.19 | | |
| Boc-Tyr-OMe | 0.24 | 0.24 | | |

[a] Injection of 1/10 amount of sample gave $k'_D = 4.26$, $k'_L = 5.52$, $\alpha = 1.29$, a shoulder peak.

be separated with an α value of 1.47 and an $R_s$ value of 0.64. For the corresponding carboxyl MIP, injection of 40 μg of Boc-D,L-Trp gave an α value of 2.03 and an $R_s$ value of 0.16. Thus, in this case the amide MIP has a capacity at least 10 times greater than the carboxyl MIP.

Since most templates used in this investigation have a free carboxyl group, we decided to imprint two templates without free carboxyl groups (Ac-L-Trp-OEt and Cbz-Ala-$NH_2$). Polymers imprinted with both compounds exhibited good enantiomeric recognition, showing that a free carboxyl group on the template is not obligatory for the polymer to exhibit good recognition (Table 8).

Two compounds other than amino acid derivatives, (*S*)-(−)-4-benzyl-2-oxazolidinone and D-(−)-mandelic acid were also imprinted. Both of the resulting polymers exhibited good enantiomeric recognition (Table 8), showing that compounds other than amino acid derivatives can also be used as templates.

**Table 6. Chromatographic Results for Enantiomeric Resolution of Structurally Related Compounds on an Amide MIP Prepared Using Cbz-L-Phe as the Template**

| test compound | $k'_D$ | $k'_L$ | α | $R_s$ |
|---|---|---|---|---|
| Boc-Trp | 2.09 | 2.09 | | |
| Boc-Tyr | 2.70 | 3.08 | 1.15 | 0.17 |
| Boc-Phe | 1.25 | 1.60 | 1.29 | 0.52 |
| Cbz-Trp | 3.39 | 4.87 | 1.44 | 0.62 |
| Cbz-Tyr | 4.54 | 7.80 | 1.72 | 0.94 |
| Cbz-Phe | 2.39 | 5.64 | 2.36 | 1.58 |
| Ac-Trp | 3.15 | 3.15 | | |
| Ac-Tyr | 4.56 | 5.02 | 1.10 | 0.10 |
| Ac-Phe | 1.89 | 2.30 | 1.22 | 0.37 |
| Ac-Trp-OEt | 0.18 | 0.18 | | |
| Boc-Tyr-OMe | 0.21 | 0.21 | | |

**Table 7. Chromatographic Results for Enantiomeric Resolution of Structurally Related Compounds on an Amide MIP Prepared Using Ac-L-Trp as the Template**

| test compound | $k'_D$ | $k'_L$ | α | $R_s$ |
|---|---|---|---|---|
| Boc-Trp | 1.09 | 1.25 | 1.16 | 0.12 |
| Boc-Tyr | 1.27 | 1.27 | | |
| Boc-Phe | 0.66 | 0.66 | | |
| Cbz-Trp | 1.76 | 2.64 | 1.50 | 0.60 |
| Cbz-Tyr[a] | 2.15 | 2.15 | | |
| Cbz-Phe | 1.16 | 1.16 | | |
| Ac-Trp | 1.73 | 5.61 | 3.24 | 2.02 |
| Ac-Tyr | 2.01 | 3.09 | 1.54 | 0.70 |
| Ac-Phe | 0.95 | 1.41 | 1.49 | 0.58 |
| Ac-Trp-OEt | 0.11 | 0.11 | | |
| Boc-Tyr-OMe | 0.11 | 0.11 | | |

[a] Injection of 1/10 amount of sample gave $k'_D = 3.62$, $k'_L = 4.18$, $\alpha = 1.16$, a shoulder peak.

**Table 8. Chromatographic Results for Enantiomeric Resolution of Amide MIPs**

| print molecule | $k'_D$ | $k'_L$ | α | $R_s$ |
|---|---|---|---|---|
| Ac-L-Trp-OEt | 0.14 | 0.25 | 1.77 | 0.49[a] |
| | 5.74 | 11.30 | 1.97 | 2.60[b] |
| Cbz-L-Glu | 2.56 | 5.10 | 1.99 | 1.54[c] |
| Cbz-L-Ala | 1.88 | 3.72 | 1.98 | 1.58[d] |
| Cbz-L-Ala-$NH_2$ | 0.59 | 1.03 | 1.75 | 1.48[e] |
| D-(−)-mandelic acid | 2.12 | 3.77 | 1.78 | 1.02[f] |
| (*S*)-(−)-4-benzyl-2-oxazolidinone | 1.18 | 1.56 | 1.32 | 1.11[g] |

[a] $CH_3CN$ was used as the mobile phase, 0.4 μg of Ac-D,L-Trp-OEt was injected in a total volume of 20 μL of mobile phase. When the mobile phase was changed to $CHCl_3$−heptane (1:1), no separation was observed. [b] Polymer was made in $CHCl_3$; $CHCl_3$−heptane (1:1) was used as the mobile phase; 10 μg of sample was injected. [c] 3% $H_2O$ in $CH_3CN$ was used as the mobile phase; 40 μg of sample was injected. [d] $CH_3CN$ was used as the mobile phase; 40 μg of sample was injected. [e] $CH_3CN$ was used as the mobile phase; 20 μg of sample was injected. [f] 0.1% HAc in $CH_3CN$ was used as the mobile phase; 20 μg of sample was injected; the flow rate was 0.5 mL/min. [g] Polymer was made in $CHCl_3$; $CHCl_3$−heptane (3:1) was used as the mobile phase; 10 μg of sample was injected.

**Linear Gradient Elution.** When using HPLC to analyze MIPs, one common problem is that the more retarded peak is normally very broad, highly asymmetric, and tailing badly. This is probably due to the fact that molecular imprinting normally cannot create homogeneous binding sites; instead there is a distribution of binding sites with different affinities for the sample molecule, as indicated by binding analysis in several cases.[12] Slow mass transfer maybe also be a reason. The tailing problem can considerably increase the time needed to complete one analysis. Bad peak symmetry makes it difficult to measure HPLC chromatogram parameters accurately. In order to distinguish the sample peak from

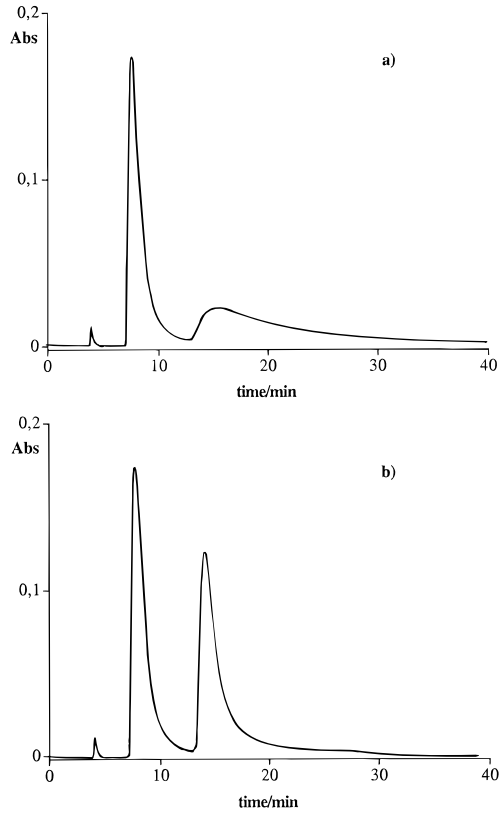



**Figure 2.** Comparison of an enantiomeric separation using isocratic or gradient elution. Amide MIP was made against Boc-L-Trp. (a) Mobile phase, 0.3% acetic acid in acetonitrile; flow rate, 1.0 mL/min; 40 $\mu$g of Boc-D,L-Trp was injected in 20 $\mu$L of acetonitrile; $k'_D = 0.92$, $k'_L = 2.83$, $\alpha = 3.08$, $R_s = 1.97$. (b) Gradient elution: solvent A, acetonitrile; solvent B, acetic acid; 0−6.71 min 0.3% B, 6.71−7.71 min 0.3−7% B, 7.71−22 min 7% B, 22−23 min 7−0.3% B, 23−40 min 0.3% B; flow rate, 1.0 mL/min; 40 $\mu$g of Boc-D,L-Trp was injected in 20 $\mu$L of acetonitrile; $k'_D = 0.94$, $k'_L = 2.53$, $\alpha = 2.69$, $R_s = 2.51$.

the noise, a competing ligand (like acetic acid) must sometimes be added to the mobile phase; this may reduce the enantiomeric separation considerably. Gradient elution has previously been used with a carboxyl MIP with marginal improvement in peak symmetry.[7b] For amide MIPs, our results showed that, by using linear gradient elution, the peak symmetry was greatly improved, the tailing problem was mostly overcome, and the time needed for one analysis was reduced. The resolution was also increased due to the increase in peak symmetry, although with some decrease in the separation value (Figure 2).

**Selectivity of Amide MIPs.** Tryptophan, tyrosine, and phenylalanine derivatives were chosen for the selectivity study. The similar structures of these amino acid derivatives should provide a good test of the polymers' selectivities. The changes in enantiomeric recognition with variation of the side chain and the protecting group and with the esterification of the carboxyl group offer good opportunities to investigate the factors that determine the selectivity of MIPs (Tables 1−7).

The amide functional group copolymerized into the polymer matrix is the main factor responsible for the enantiomeric recognition. For the amide MIP made against Boc-L-Trp, addition of a competing ligand (acetic acid) dramatically reduced the enantiomeric recognition (Figure 3). When 15% acetic acid was used, no enantiomeric separation was observed. This is because acetic acid competed with the sample molecule for the amide hydrogen-bonding functional groups and reduced the possibility of hydrogen-bonding interactions between the sample molecule and the amide groups at the recognition sites.

For any enantiomeric recognition, at least three of the four groups around the chiral center must be specifically recognized. For amino acid derivatives, these three groups are the carboxyl group, the amino protecting group, and the side chain.

(a) We believe that, prior to polymerization, a complex forms between the free carboxyl group of the templates and the amide group of the functional monomer (Schemes 1−3). Esterification of the template carboxyl group prevents the formation of hydrogen bonds between this group and the amide group of the polymer (Scheme 2b). Neither of the two esters, Ac-D,L-Trp-OEt or Boc-D,L-Tyr-OMe, could be separated by the MIPs made against templates with a free carboxyl group. The amount of sample injected onto the MIPs made against Ac-L-Trp and Boc-L-Tyr was 0.8 $\mu$g, only 2% of the amount of sample normally injected, and still no enantiomeric recognition was observed. In fact, the ester enantiomers were barely retarded.

Similarly none of the enantiomers except Ac-D,L-Trp-OEt could be separated by an amide MIP made against Ac-L-Trp-OEt.

(b) The protecting groups (acetyl, Boc, and Cbz groups) are all capable of forming hydrogen bonds. The acetyl derivative itself contains an amide functionality. Since the template, (*S*)-(−)-4-benzyl-2-oxazolidinone (which possesses only a carbamate group) could be imprinted and gave good enantiomeric recognition, the carbamate group is clearly capable of forming hydrogen bonds. Both Boc and Cbz groups contain a carbamate functional group. The most obvious difference among these three protecting groups is their size: the acetyl group is much smaller than the other two. Also, the Cbz group is a flat structure while the Boc group is more three-dimensional.

For all of the amide MIPs made against Trp, Tyr, and Phe derivatives, better enantiorecognition was always observed for compounds with the same protecting group as the template than for compounds with different protecting groups. Better enantioseparation was observed for Cbz derivatives on amide MIPs made against Boc derivatives than for Boc derivatives on amide MIPs made against Cbz derivatives. This is because the Boc group has a more three-dimensional structure, the cavity created by imprinting the Boc group would be bigger than the cavity created by imprinting the Cbz group, such that the Boc group created cavity could accommodate the Cbz group better than the Cbz group created cavity accommodated the Boc group (Scheme 1c). For the amide MIP made against Ac-L-Trp, the relatively poor enantioseparation of the Boc and Cbz derivatives may be attributed to the smaller size of the acetyl group compared with the Boc and Cbz groups (Scheme 3). In this case also, Cbz-D,L-Trp was better separated than Boc-D,L-Trp. The results showed that, except for the amide MIP made against Ac-L-Trp, acetyl derivatives were generally only poorly separated by other amide MIPs. This may be also due to the smaller size of the acetyl group. Besides the "correct" interaction with the recognition sites, acetyl derivatives could more easily fit "incorrectly" and form stronger nonspecific interactions, for instance between the acetyl group and the amide that

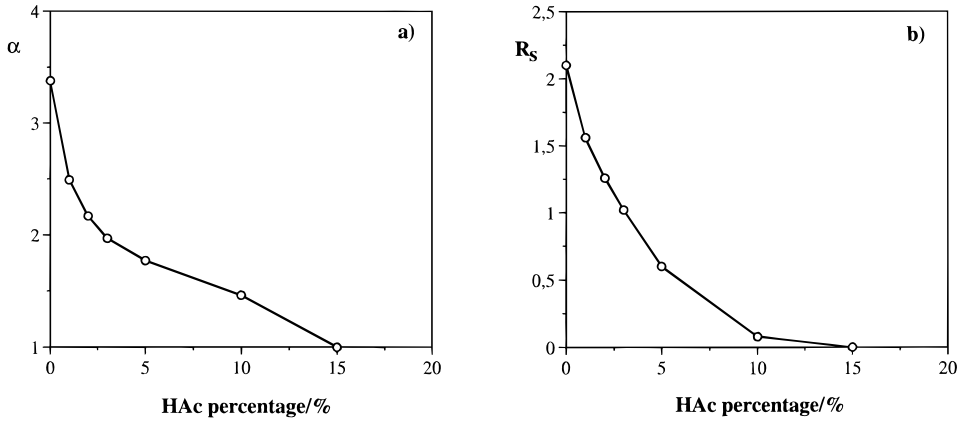
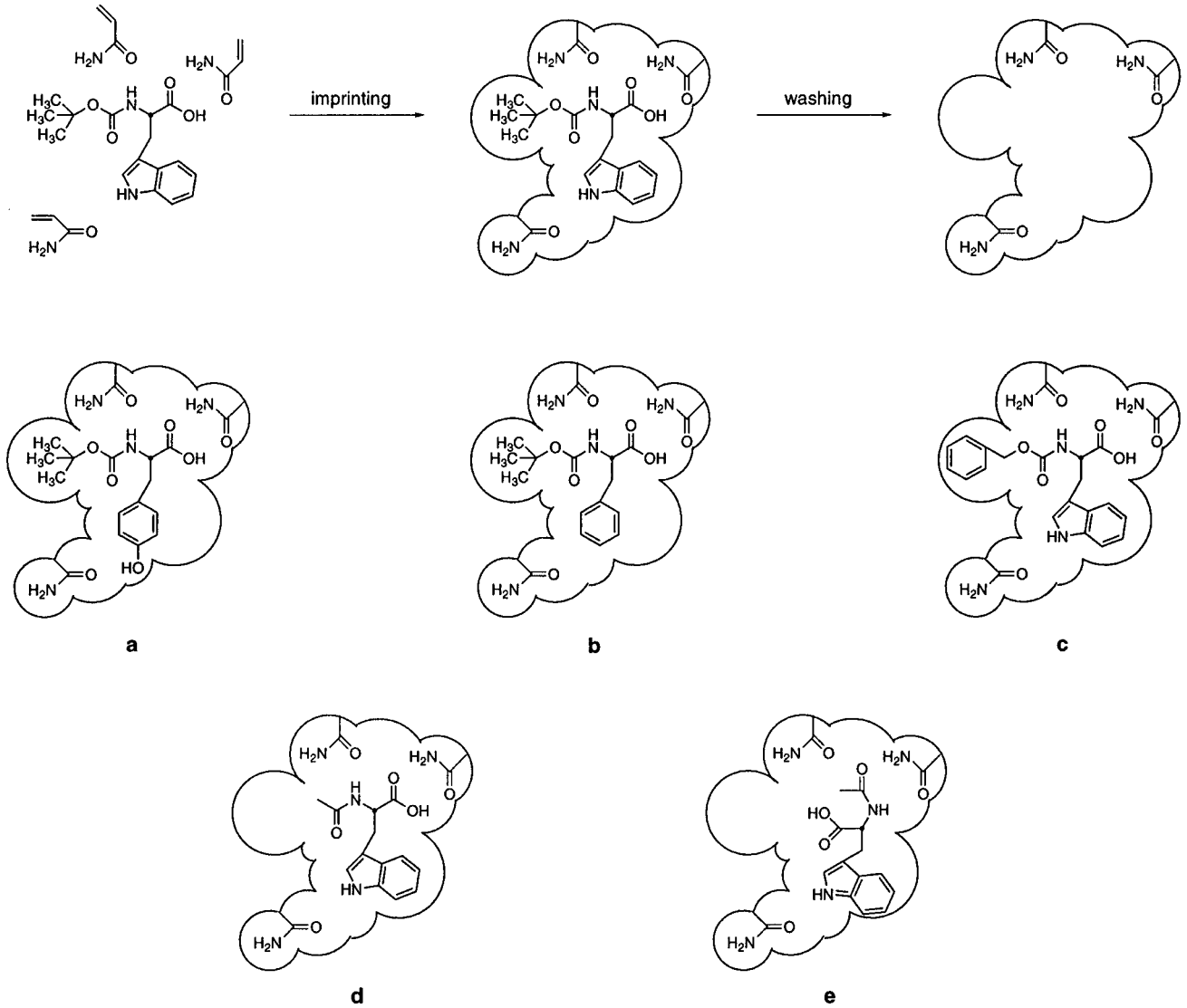



**Figure 3.** Enantiomeric recognition (α and $R_s$) versus acetic acid concentration in the mobile phase. Amide MIP was made against Boc-L-Trp. Mobile phase: acetonitrile−acetic acid; flow rate, 1.0 mL/min; 40 μg of Boc-D,L-Trp was injected in 20 μL of acetonitrile.

**Scheme 1**

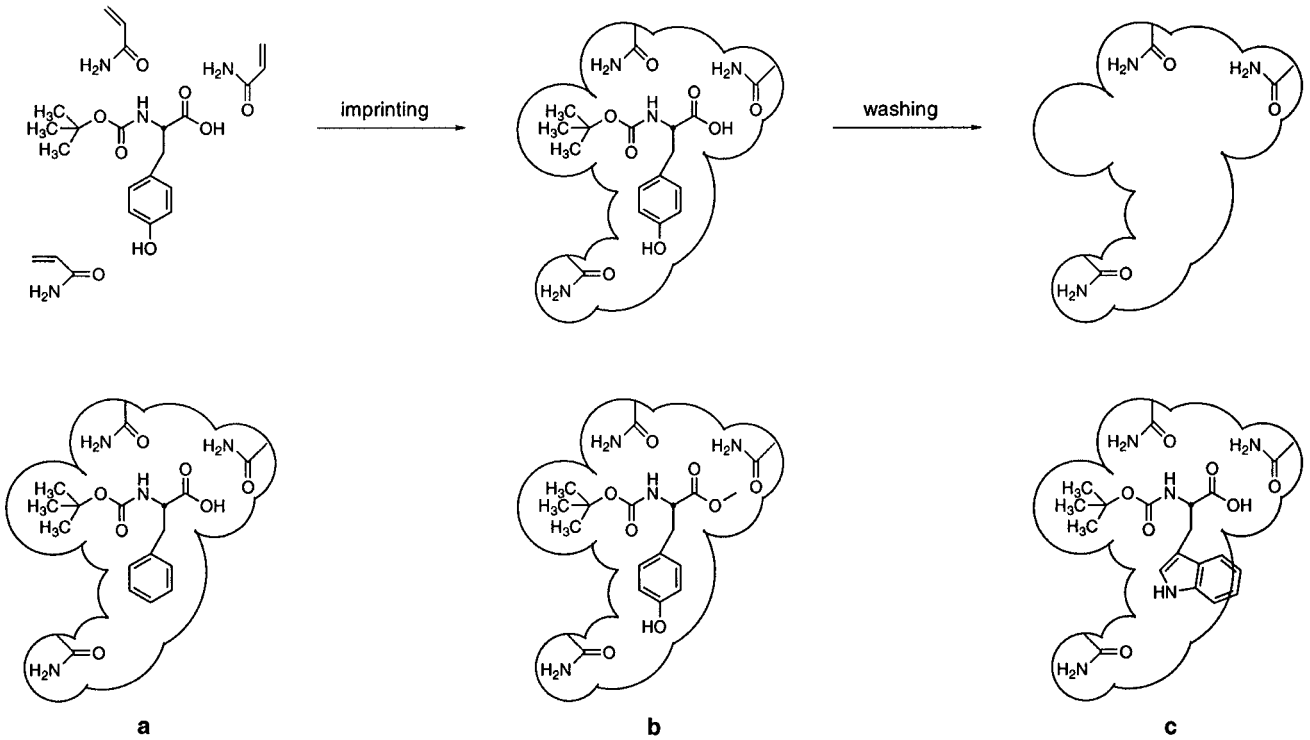



should interact with the free carboxyl group, making the separation less effective (Scheme 1d,e).

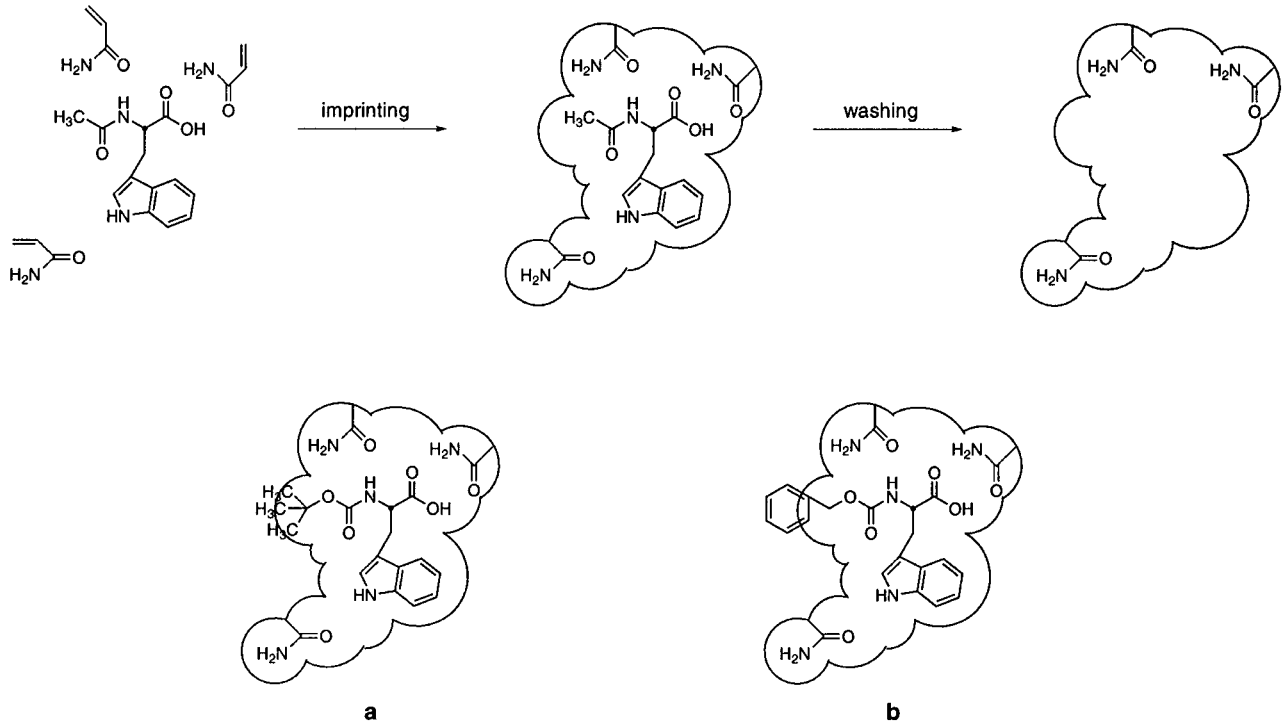
(c) The side chains of tryptophan and tyrosine can form hydrogen bonds with the amide group while the phenyl group of phenylalanine cannot. This is supported by the fact that, for the D-enantiomers, the capacity factors of the Trp and Tyr derivatives on all amide MIPs are always larger than those of the Phe derivatives, indicating that there are additional nonspecific hydrogen-bonding interactions. The phenyl group and the hydroxyphenyl group are quite similar in size; the indole group of tryptophan on the other hand is much larger.

For amide MIPs imprinted against Trp derivatives, the polymers always gave better separation for Tyr deriva-

**Scheme 2**




**Scheme 3**




tives than for Phe derivatives. This may be due to the fact that Tyr derivatives could hydrogen bond with the amide group in the cavity created by the indole group (Scheme 1a,b). However, MIPs made against Tyr derivatives exhibited better separation of Phe derivatives than Trp derivatives, and amide MIPs made against Phe derivatives exhibited better separation of Tyr derivatives than Trp derivatives. This is because the cavity created by the hydroxyphenyl group or the phenyl group was not big enough to accommodate the indole ring readily (Scheme 2a,c). Amide MIPs made against Phe derivatives exhibited better selectivity for Phe derivatives than for Tyr derivatives. Thus, a single hydroxy group can clearly contribute to the recognition exhibited by amide MIPs.

For templates with more hydrogen-bonding sites available, the resulting MIPs normally showed better separation of the enantiomers of the template. Amide MIPs made against tryptophan and tyrosine derivatives always gave better enantiomeric recognition of the print compounds than amide MIPs made against phenylalanine derivatives.

Besides all the above discussions about selectivity, perhaps the most obvious demonstration of the selectivity

of all the amide MIPs is that the racemate of the print molecule was always better separated than any other racemic pair.

**Enantiomeric Recognition in Water.** As a continuation of this research, we are also investigating enantiomeric recognition by amide MIPs in aqueous systems. Preliminary results show that amide MIPs can also demonstrate good enantiomeric recognition in water. For example, for the amide MIP made against Boc-L-Trp, when the mobile phase was chosen to be water−acetonitrile (7:3), 10 mM glycine buffer, pH = 3.0, 10 $\mu$g of Boc-D,L-Trp could be separated with $\alpha = 1.74$ and $R_s = 2.15$. Our results also show that the enantiomeric separation changes with changes of mobile phase water percentage, pH, salt concentration, etc. We believe that the hydrophobic effect makes a major contribution to recognition in aqueous media.

## Conclusions

As a new hydrogen-bonding functional group, amide groups were introduced into molecularly imprinted polymers via copolymerization of ethylene glycol dimethacrylate with acrylamide in the presence of different templates. We assume that strong hydrogen-bonding interactions were formed between the template and the amide functional groups in a polar organic solvent (acetonitrile) prior to polymerization. The resulting polymers showed much improved enantiomeric recognition and load capacities compared to similarly prepared carboxyl MIPs. By using linear gradient elution, the peak symmetry was much improved, and the peak-tailing problem was mostly overcome. We ascribe the selectivity of amide MIPs to the hydrogen bonds being formed between the sample molecule and amide groups at the recognition sites of the imprinted polymer and to the size and shape of the sample molecule. Further studies are planned to elucidate more in details if and to what degree other forces apart from hydrogen bonding are involved in the recognition of amide MIPs. The amide MIPs were also capable of enantioseparation in aqueous media.

In summary, a new technique has been added to the field of molecular imprinting utilizing noncovalent interactions for the preparation and application of the polymers. This method leads to better enantiorecognition of the compounds studied, in particular those containing free carboxylic groups, and, at least as important, allows the preparation of synthetic polymers without the excess of charged groups which often leads to the problems of swelling and nonspecific binding.

## Experimental Section

Amino acid derivatives were obtained from Sigma Chemical Co. (St. Louis, MO), Nova Biochem (Läufelfingen, Switzerland), or Bachem (Bubendorf, Switzerland). Methacrylic acid and ethylene glycol dimethacrylate (EGDMA) were from E. Merck (Darmstadt, Germany). 2,2′-Azobisisobutyronitrile (AIBN) was from Janssen Chemica (Beerse, Belgium). Acrylamide was from Bio-Rad (Richmond, CA). Acetonitrile and chloroform were of HPLC grade.

**Polymer Synthesis.** Polymers were prepared using acrylamide as the functional monomer and EGDMA as the cross-linker. The molar ratio of print molecule to functional monomer to cross-linker was 1:4:20 expect for (*S*)-(−)-4-benzyl-2-oxazolidinone; due to there being less hydrogen-bonding sites available on this template, a molar ratio of 1:2:20 was used instead. Generally polymers were synthesized using 10 g of EGDMA, 100 mg of AIBN (the free radical initiator), and the correct amount of acrylamide and template. The mixture was dissolved in 15 mL of acetonitrile (or chloroform for (*S*)-(−)-4-benzyl-2-oxazolidinone and one amide MIP made against Ac-L-Trp-OEt), degassed in a sonicating water bath, saturated with nitrogen for 5 min, and polymerized under UV irradiation (366 nm) at 4 °C for 24 h. The polymer was then ground in a mechanical mortar (Retsch, Haan, Germany), sieved through a 25 $\mu$m sieve and fines were removed by repeated sedimentation in acetone.

**HPLC Analysis.** After sedimentation, particles were suspended in acetone and slurry packed into 250 mm × 4.6 mm i.d. stainless-steel columns at 30 MPa using an air-driven fluid pump and acetone as solvent. An average of 1.47 g of polymer could be packed into the column under these conditions (21 different experiments). The column was washed on-line with methanol−acetic acid (9:1, v/v) until a stable baseline was achieved.

Generally, within the detection limit, the mobile phase was chosen to give good enantiomeric separation in a reasonable period of time. For the selectivity study, pure acetonitrile was used as the mobile phase. Although in several cases, especially for tyrosine (Tyr) derivatives, the retention times were very long, and this made HPLC analyses quite time consuming, no competing solvent like acetic acid was added to the mobile phase to reduce the retentions. Changing the mobile phase can change the retention time, the separation factor, and the resolution value considerably; this would invalidate the comparison between different polymers.

For all of the HPLC analyses performed in this investigation, unless specified, normally a 40 $\mu$g sample dissolved in 20 $\mu$L of acetonitrile was injected and analyzed isocratically at a flow rate of 1.0 mL/min using acetonitrile as the mobile phase. For the compounds analyzed in the selectivity study, if a racemate could not be separated by a particular amide MIP, normally 1/10 the amount of sample was also analyzed (the result is given as a footnote if it was separated).

Acetone was used as the void marker. Capacity factors ($k'_D$ and $k'_L$), separation factors ($\alpha$), resolutions ($R_s$), and plate numbers ($N$) were all calculated according to standard chromatographic theory.[34] For instance, $k'_D = (t_D - t_0)/t_0$, $k'_L = (t_L - t_0)/t_0$, $\alpha = k'_L/k'_D$, where $t_D$ is the retention time of the D enantiomer, $t_L$ is the retention time of the L enantiomer, $t_0$ is the retention time of the void marker. The plate number ($N$) for acetone for the amide MIP made against Boc-L-Trp was determined to be 1508.

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(34) Poole, C. F.; Schuette, S. A. *Comtemporary practice of chromatography*; Elsevier: Amsterdam, 1984.