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A Substructure Approach toward Polymeric Receptors Targeting Dihydrofolate Reductase Inhibitors. 2. Molecularly Imprinted Polymers against *Z*-L-Glutamic Acid Showing Affinity for Larger Molecules

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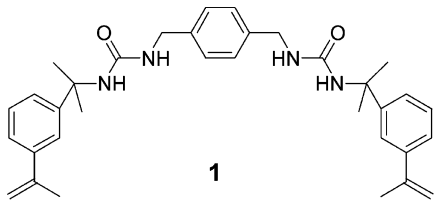
Abstract: The preparation of a molecularly imprinted polymer against *N*-*Z*-L-glutamic acid using a novel bis-urea functional monomer is described. The polymer exhibits affinity for the template over *N*-*Z*-protected aspartic acid and glycine and, further, is capable of binding larger molecules, e.g., the anti-cancer drug methotrexate, containing the glutamate substructure.

The use of molecular imprinting, as a method to prepare materials showing strong and selective affinity for the imprinted (templated) structure and closely related analogues, has received growing interest in recent years.[1] The most flexible approach to imprinting is the "noncovalent" approach,[2] depicted schematically in Figure 1, where template-functional monomer assemblies, formed by noncovalent interactions, are "locked" into a three-dimensional network on co-polymerization with an excess of cross-linking monomer. Extraction of the template from the polymer matrix leaves behind sites which are complementary in shape and functional to the template (and analogues).

This approach is very attractive due to its inherent simplicity, but problems remain, notably with regards to the imprinting of templates which are not soluble in the low-polar organic solvents generally used in the imprinting step, e.g., chloroform and toluene. A further problem is that the use of commercially available functional monomers generally requires that a large excess of said monomers are employed to ensure that the template molecule is completely complexed. This, in turn, means that nonassociated functional monomer is incorporated into the polymer matrix, leading to high levels of nonspecific binding.

FIGURE 1. Highly schematic representation of the strategy of molecular imprinting.

The use of designed functional monomers in molecular imprinting is not common, although there have been notable exceptions.[3−7] Herein, we wish to report the synthesis of the novel, functional monomer **1** and its application in the imprinting of *N*-*Z*-L-glutamic acid (**2**). Further, as a continuation of an earlier study on substructure approaches to the imprinting of dihydrofolate reductase inhibitors,[8] we wish to report the recognition properties of the polymers toward larger molecules containing the glutamate substructure, e.g., the anticancer drug methotrexate (**3**).



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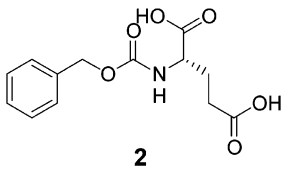

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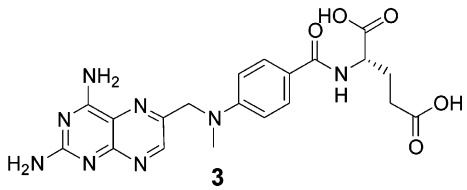

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Our design for the novel functional monomer reported herein is based on a report by the group of Hamilton,[9] where a suitably spaced bis-urea was found to exhibit reasonable affinity for glutaric acid (as its bis-tetrabu-

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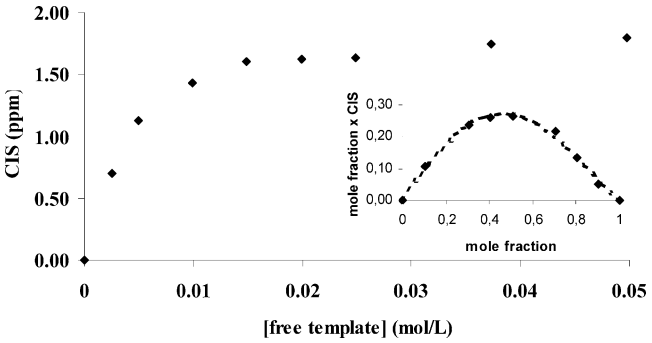

FIGURE 2. ^{1}H NMR titration data for titration of **1** with bis-TBA-glutarate in DMSO-d_6. The CIS of the "outer" urea proton is shown The inset shows the corresponding Job plot.

tylammonium (TBA) salt) in a competitive medium (K_a = 790M^{-1} via ^{1}H NMR titration at 20 °C in DMSO-d_6; K_a = 1300 M^{-1} via isothermal titration calorimetry at 25 °C in DMSO).

The polymerizable bis-urea receptor (**1**) was readily prepared in one step, from *p*-xylylene diamine and 3-isopropenyl-α,α-dimethylbenzyl isocyanate, in 67% yield. To determine the binding abilities of (**1**), we performed ^{1}H NMR titrations using bis-TBA-glutarate as our model dicarboxy anion guest (see Figure 2).[10] Addition of increasing amounts of bis-TBA glutarate (0−10 equiv) to DMSO-d_6 solutions of **1** (concentration = 5 mM) led to significant downfield complexation-induced shifts (CIS) of both the "inner" and "outer" urea proton signals (CIS ≈ 1.8 ppm in each case). A Job plot titration (inset Figure 1), performed to establish the stoichiometry of the interaction revealed a small deviation from the expected 1:1 monomer/guest stoichiometry (maximum at mole fraction **1** ≈ 0.45). Indeed, the data obtained in the first titration exhibited deviation from the theoretical 1:1 binding isotherm at higher guest concentrations (7.5 and 10 equiv); this is indicative of the formation of higher order complexes. Exclusion of the deviant points led to a good fit to a 1:1 binding isotherm, and an apparent association constant (K_{app}) of 1500 M^{-1} was extracted.

Having proven that **1** interacts strongly with the model dicarboxy anion, it was used in the preparation of a MIP using bis-TBA-*N*-*Z*-L-glutamate (prepared by the reaction of *N*-*Z*-L-glutamic acid with 2 equiv of TBA hydroxide in methanol) as the template and DMSO as the polymerization solvent. Thermally initiated polymerization at 40 °C was allowed to continue for 24 h. Following the polymerization, the template was removed by extraction with methanol in a Soxhlet apparatus. The polymer was then crushed and sized to yield particles of size 25−50 μm. A control, nonimprinted polymer (NIP) was prepared under identical conditions, but with the omission of the template molecule.

The recognition properties of the respective polymers toward the template, its optical antipode, and other amino acids were examined via HPLC using mobile phases based on acetonitrile (ACN) and modified with triethylamine (TEA). The results are summarized in Figure 3.

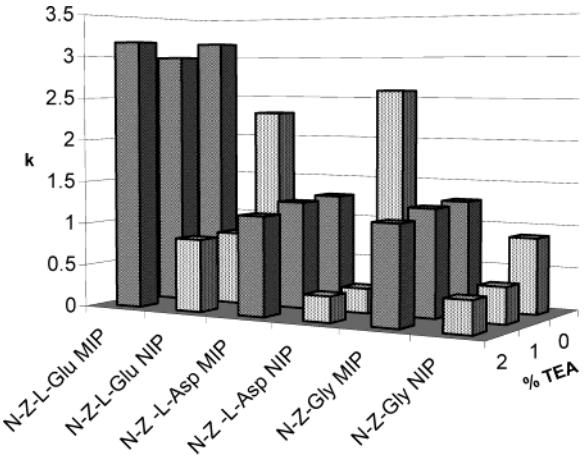

FIGURE 3. Retention behavior of the template and related analytes on the imprinted (MIP) and nonimprinted (NIP) polymers in TEA-modified ACN mobile phases. The retention of *N*-*Z*-D-Glu was identical to that of *N*-*Z*-L-Glu, within experimental error. Retention is expressed as the retention factor $k = (t(\text{analyte}) - t_0)/t_0$, where t(analyte) and t_0 are the retention times of the analyte and the void volume marker (acetone), respectively. Data calculated as the average of at least three injections with an RSD < 4%. Conditions: 20 μL injections of analytes (10 mM), flow rate = 1 mL/min, column dimensions = 100 mm × 4.6 mm i.d., detection at 260 nm.

Considering first the behavior of the template, we see that with pure acetonitrile as the mobile phase the differences in retention between the MIP and NIP are relatively small. This is not overly surprising, as the urea functionality should bind only weakly to nondissociated acid functions. Modification of the mobile phase with small amounts of TEA (1−2%) leads to the observance of a definite imprinting effect; this effect levels off after addition of 2% TEA to the mobile phase. These observations act as a further confirmation of the mode of imprinting and subsequent recognition in these polymers, i.e., that the urea functionalities interact strongly with template only after the addition of an agent capable of causing deprotonation. Unfortunately, we found that the MIP exhibited no enantioselectivity. Polarimetry measurements indicated that this was not due to racemisation of the template during any of the preparation procedures. The binding site was designed to be able to accommodate 1,5-diacids primarily, while leaving space for larger substituents at N. Thus, we used a stoichiometric amount of **1** to complement the acid groups in the template. For enhanced enantioselectivity, according to Dalgleish's three-point rule,[11] three interactions are required for effective enantiodiscrimination. This may be achieved in the present case by targeting the carbamate moiety, but was not the objective in the present work.

Despite the lack of enantioselectivity observed, we found that the MIP was able to separate **2** from an equimolar mixture with *N*-*Z*-L-Asp and *N*-*Z*-Gly, as shown in Figure 4, while the NIP was not. The retention order of the analytes on both polymers (single injections)

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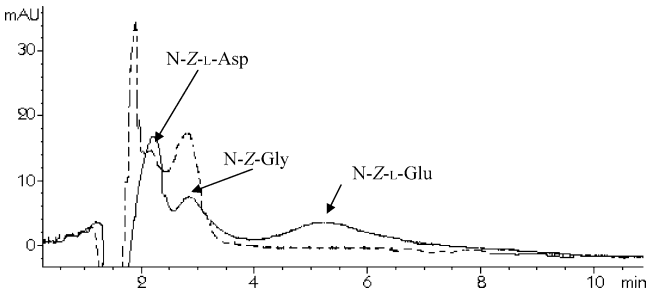
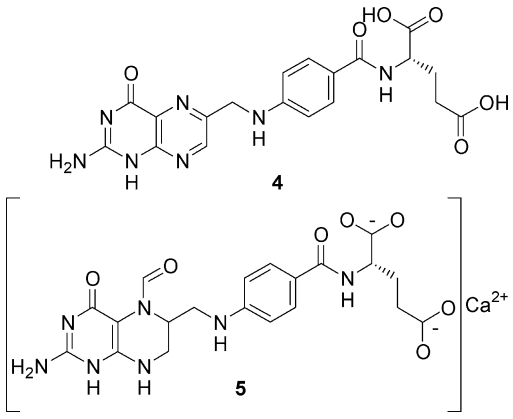


FIGURE 4. Separation of an equimolar mixture of *N*-*Z*-L-Glu (**2**), *N*-*Z*-L-Asp, and *N*-*Z*-Gly (each 3.33 mM) on the MIP (solid line) and NIP (broken line) columns. Conditions: mobile phase 2% TEA in ACN, flow rate = 1 mL/min, column dimensions = 100 mm × 4.6 mm i.d., detection at 260 nm.

follows a similar order, perhaps reflecting the predisposition of the functional monomer toward 1,5-dicarboxy anions. However, retention of **2** is much stronger on the MIP, suggesting that the imprinting process enhances this predisposition by "locking in" the functionality of the monomer and reducing the inherent flexibility of the monomeric "binding pocket".

As a continuation of our earlier work on substructure imprinting approaches for the recognition of dihydrofolate reductase inhibitors,[8] we were further interested in assessing the ability of the MIP to recognize larger molecules containing the glutamate substructure, e.g., methotrexate (**3**), folic acid (**4**), and leucovorin (**5**).



The three molecules exhibit vastly different behavior when applied to the MIP and NIP columns. In all three mobile phases, **3** is retained much more strongly on the MIP than on the NIP, the retention increasing with the amount of TEA in the mobile phase (see Figure 5). Conversely, both **4** and **5** eluted either at the same time or before the void marker (acetone).

In the case of **3**, the basicity of the pteridine part of the molecule is likely to be sufficient to cause at least some deprotonation of the glutamate moiety, thus leading to retention of **3** even in the unmodified ACN mobile phase. The dissociated state is ensured in the TEA-modified mobile phases and, accordingly, leads to increased retention of **3**. The enhanced recognition of **3** by the MIP when compared to the NIP provides further evidence for the success of the imprinting process. The behaviors of **4** and **5** on both MIP and NIP columns are somewhat more difficult to explain. The glutamate portion of **3** is reported to have pK_a values of 3.4 and 4.7,[12] while for the acid form of **5** (folinic acid) the values are 3.1 and 4.8.[12] In our study, **5** is present as the calcium salt. Thus, for **5** to bind effectively to the polymers, the binding energy needs to overcome that of the calcium complex; we feel that this is unlikely to occur, hence the elution of **5** without retention on the MIP and NIP columns. No pK_a values are available for the glutamate portion of **4**, as it is soluble only between pH 5−10.5.[13] While it is reasonable to assume that the glutamate portion of **4** has pK_a values similar to those of **3**, we attribute its lack of retention on the polymers to the nature of the pteridone substructure of the molecule, although the reason is not fully clear.

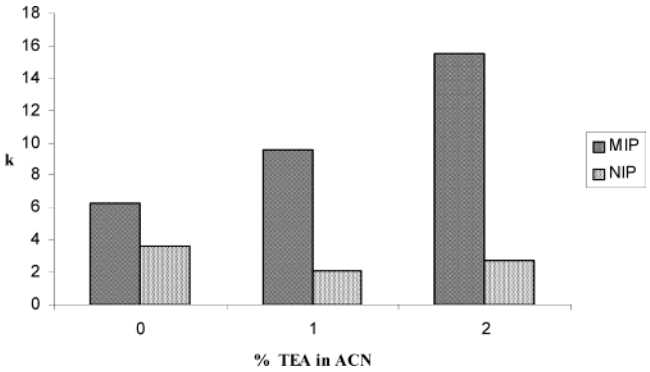


FIGURE 5. Retention of methotrexate (**3**) on the imprinted and nonimprinted polymers in different mobile phases. The retentions are given as retention factors, *k*, as defined in Figure 2. Conditions: 20 μL injections of **3** (1 mM), flowrate = 1 mL/min, column dimensions = 100 mm × 4.6 mm i.d., detection at 260 nm.

Conclusions

A novel bis-urea functional monomer has been prepared, and its ability to form strong intermolecular hydrogen bonds to 1,5-dicaboxy anions in a competitive medium (DMSO-d_6) has been quantified. In the subsequently prepared MIP, imprinting effects are observed only when the analyte is in the deprotonated state, thus confirming the mode of binding to the pendant polymer functionality. The MIP is selective for *N*-*Z*-L-Glu over *N*-*Z*-L-Asp and *N*-*Z*-Gly. Further, the MIP is able to bind the drug methotrexate, thus providing further evidence that substructure approaches to molecular imprinting offer a viable alternative to the imprinting of the entire target structure. This may be important in cases where the target exhibits limited solubility or is prohibitively expensive. Due to the predisposition of the bis-urea monomer to bind α,ω-C_5 diacids, its use in combination with competing monomers to imprint larger targets containing the glutamic acid substructure may also be feasible. This is the subject of our current investigations.

Experimental Section

Materials and Methods. All materials were of reagent grade unless otherwise stated. 3-Isopropenyl-α,α-dimethylbenzyl isocyanate and *N*-*Z*-L-glutamic acid (**2**, 99%) were purchased from

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Aldrich (Deisenhofen, Germany), while *N*-*Z*-D-glutamic acid (98+%) was from Lancaster (Morecombe, UK). 1,4-Xylylenediamine, glutaric acid, 1 M methanolic tetrabutylammonium hydroxide, and tetrabutylammonium benzoate were purchased from Acros Organics (Geel, Belgium). Methotrexate (**3**, (+)-amethopterin, >98%), folic acid (**4**, 98%), leucovorin (**5**, *dl*-L-form of folinic acid, as its calcium(II) salt, 95%), *N*-*Z*-L-aspartic acid (99%), and *N*-*Z*-glycine (99%) were purchased from Sigma (St. Louis, MO). Anhydrous solvents, tetrahydrofuran and dimethyl sulfoxide, were obtained from Fluka (Deisenhofen, Germany) and stored over appropriate molecular sieves. All solvents used in the chromatographic experiments were of HPLC grade and were purchased from Carlo Erba (Milan, Italy).

All NMR spectra were obtained using a Bruker Advance DRX 400 spectrometer. ^{1}H spectra were obtained at 400 MHz, and ^{13}C spectra were obtained at 100 MHz. Elemental microanalysis was performed using a "CHN-rapid" HERAEUS analyzer (Hanau, Germany).

All HPLC evaluations were performed using a HP1090 instrument (Hewlett-Packard, Palo Alto, CA) equipped with a Rheodyne sample valve (20 mL loop) and a diode array detector, connected to a HP ChemStation (Version A.06.03). Mobile phases were degassed by sparging with He for 10 min prior to column conditioning. All analyses were conducted at room temperature at a flow rate of 1 mL/min, with detection at 260 nm. Retention factors are expressed as $k = (t - t_0)/t_0$, where t is the retention time of the analyte and t_0 is the retention time void marker (acetone). Analyte sample preparation was as follows: *Z*-protected amino acids were all as solutions in ACN; for **3**, a 5 mM solution in *N*-methylpyrrolidinone (NMP) was diluted to 1 mM with ACN; a 1 mM solution of **4** was prepared in NMP; a 1 mM solution of **5** was prepared in water.

Synthesis of 1-[1-(3-Isopropenylphenyl)-1-methylethyl]-3-[4-[3-[1-(3-isopropenylphenyl)-1-methylethyl]ureidomethyl]benzyl]urea (1). 1,4-Xylylene diamine (2.72 g, 20 mmol) was dissolved in anhydrous tetrahydrofuran (125 mL), and the solution was stirred at ambient temperature under a stream of nitrogen. 3-Isopropenyl-α,α-dimethylbenzyl isocyanate (8.85 mL, 44 mmol) was then added dropwise, such that the heat of reaction did not cause the solvent to boil. The solution was stirred overnight at ambient temperature under a stream of nitrogen, whereafter the solvent was *removed in vacuo*. The residue was recrystallized from ethanol to yield the product as a white, crystalline solid (6.87 g, 67%): mp 210−211 °C; ^{1}H NMR (DMSO-d_6) δ 1.51 (s, 12H), 2.06 (s, 6H), 4.08 (d, 4H), 5.04 (s, 2H), 5.32 (s, 2H), 6.20 (t, 6.20, 2H), 6.35 (s, 2H), 7.09 (s, 2H), 7.19−7.26 (m, 6H), 7.44 (s, 2H); ^{13}C NMR (DMSO-d_6) δ 21.85, 30.43, 42.53, 54.43, 112.52, 122.02, 123.06, 124.5, 127.01, 128.09, 139.50, 140.28, 143.33, 149.35, 157.29; HRMS calcd 538.3308, found 538.3284. Anal. Calcd for $C_{34}H_{42}N_4O_2 \cdot 0.5H_2O$: C, 74.56; H, 7.91; N, 10.23. Found: C, 74.6; H, 7.7; N, 10.1.

Synthesis of Bis-tetrabutylammonium-*N*-*Z*-L-glutamate. *N*-*Z*-L-Glutamic acid (0.563 g, 2 mmol) was dissolved in methanol (50 mL) and 1 M methanolic tetrabutylammonium hydroxide (4 mL, 4 mmol) was added in one portion. The solution was stirred at ambient temperature for 1 h, and then the solvent was removed in vacuo. The oily residue was dried overnight at 80 °C in vacuo and was used without further purification.

Synthesis of Bis-tetrabutylammonium Glutarate. This compound was prepared from glutaric acid by the procedure described above and used without further purification.

Association Constant Determination. The model compound used as titration partner against **1** was bis-tetrabutylammonium glutarate, prepared as described above. The stoichiometry of the interaction between **1** and bis-TBA-glutarate was determined by the method of continuing variance (Job plot). Thus, equimolar solutions (5 mM) of each monomer and the glutarate in DMSO-d_6 were added together in the ratios 0:1, 0.1:0.9, 0.2:0.8, ..., 0.0.8:0.2, 0.9:0.1, and 1:0, respectively, and plots of $\Delta\delta \cdot a$ versus a were constructed, where $\Delta\delta$ refers to the complexation induced shift of the urea protons in the monomer and a is the mole fraction of monomer. Association constants for the interactions between **1** and bis-TBA-glutarate were determined by titrating an increasing amount of **2** into a constant amount of host. The concentration of **1** was 5 mM, and the amounts of glutarate added were 0, 0.5, 1, 2, 3, 4, 5, 7.5, and 10 equiv (i.e., 0−50 mM), respectively. The CIS of both sets of urea protons were monitored, and titration curves were constructed. The raw titration data were fitted to a 1:1 binding isotherm and the association constants were obtained by least-squares fitting of the isotherms using Microcal Origin.

Polymer Preparation. The molecularly imprinted polymer (MIP) was prepared as follows. Bis-tetrabutylammonium-*N*-*Z*-L-Glu (0.281 g, 1 mmol), functional monomer **1** (0.538 g, 1 mmol), ethyleneglycol dimethacrylate (3.96 g, 20 mmol), and the initiator ABDV (45 mg, 1% w/w total monomers) were dissolved in anhydrous DMSO (5.6 mL). The solution was transferred to a glass tube and then degassed by purging with nitrogen for 15 min. Polymerization was initiated by placing the stoppered tube in a water bath thermostated at 40 °C and allowed to continue for 48 h. After this time, the tubes were broken and the polymers crushed lightly. The crushed polymers were extracted with methanol in a Soxhlet apparatus for 21 h. The extracted polymers were then further crushed and sieved, and particles of size 25−50 μm were collected. This fraction was then repeatedly sedimented to remove fine particles using methanol/water (80/20 v/v), and the sedimented particles were used for the chromatographic evaluation. A control, nonimprinted polymer (NIP) was prepared in exactly the same manner, but with the omission of the template molecule from the pre-polymerization mixture. For the HPLC evaluation, the MIP and NIP were each slurry-packed into stainless steel columns (100 mm × 4.6 mm i.d., Alltech, Milan, Italy) using MeOH/water (80/20 v/v) as pushing solvent using Haskel air drive fluid pump (Haskel Inc., Burbank, CA).

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