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# Prevalence of steric restrictions in enzymatic nitrile-hydrolysis of a preparation from *Rhodococcus sp.* 409

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**Abstract**

The size of the binding pocket of a nitrilase from *Rhodococcus sp.* 409 has been probed with 25 compounds and a basic active site model of potential predictive value has been established delineating the minimum pocket dimensions within a 4 Å distance from the nitrile nitrogen atom. The total volume of this section of the model comprises 227.9 Å$^3$. Differential volume calculations were found to be indicative for hydrolysis and consistently, SYBYL CoMFA steric field reflects 70% of explained variance.

*Keywords:* Nitrilase; Nitrile hydrolysis; Active-site model; *Rhodococcus sp.* 409

## 1. Introduction

Nitrilases are useful biocatalysts for the convenient hydrolysis of organic nitriles under mild conditions [1–7]. This is of considerable synthetic value, in particular, for the modification of highly functionalized compounds [8].

Besides nitrilase activity, the immobilized whole cell preparation derived from *Rhodococcus sp.* (NOVO SP 409) contains hydratase, esterase, amidase, as well as epoxide hydrolase activity [9,10]. The enzyme system processes a wide variety of nitrile containing compounds and is capable of hydrolyzing substrates in suspension. In light of the lack of X-ray structure and even sequence information, our intention was to define prerequisites for enzymatic conversion with the aid of molecular modelling. In general, several factors can determine the turnover of substrates by enzymes. For example, electronic effects are likely to modulate the initial rate constant and the total yield of the reaction. Substituent effects on the hydrolysis of benzyl cyanides have recently been investigated [11]. However, these features are inadequate to explain the lack of hydrolysis observed for some nitriles [12]. After surveying all the substrates studied in the literature, no obvious common pattern of substitution or functional groups could be identified as essential for specific enzyme–substrate interactions and thus for hydrolysis. This stimulated us to test the hypothesis that an appropriate molecular volume could be the predominant factor necessary for conversion. To

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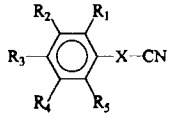
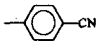
this end, 25 compounds were tested and differences in molecular volume analyzed to establish a model capable of forecasting whether the SP 409 preparation would hydrolyze specific nitriles. This hypothesis was probed by a CoMFA with the steric field descriptor.

## 2. Results and discussion

To probe the steric requirements for enzymatic hydrolysis, substrates varying in framework, substituents and substitution pattern (Tables 1 and 2) were subjected to treatment with

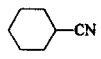
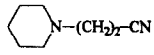
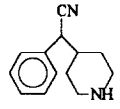
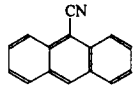
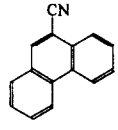
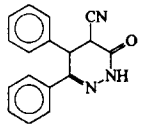
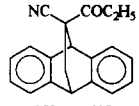
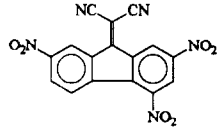
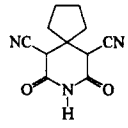
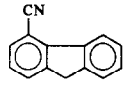
Table 1
Enzymatic hydrolysis of nitriles




| Lit[a] | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | X | Yield (%) Lit[b] | log P[c] | Vol.[d] (Å$^3$) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | H | H | H | H | H | $[CH_2]_2$- | 40 | 1.710 | - |
| 2 | $OCH_3$ | H | H | H | $OCH_3$ | - | 88 | 2.101 | - |
| 3 | $OCOCH_3$ | H | H | H | H | - | 79[e] | 1.961 | - |
| 4 Lit [12] | H | t.-but | OH | t.-but | H | $CH_2$- | - | 4.300 (3.752) | 36.7 |
| 5 Lit [12] | H | t.-but | OH | t.-but | H | $[CH_2]_2$- | - | 4.840 (4.163) | 74[f] |
| 6 Lit [12] | H | t.-but | OH | t.-but | H | $[CH_2]_2$-CO-NH-$[CH_2]_2$ | - | 4.130 | 162.4[f] |
| 7 | H |  | H | H | H | - | 64 Lit [13] | 4.970 | - |
| 8 | H | H |  | H | H | - | 72 | 2.730 | - |
| 9 | H | H |  | H | H | $CHCH_3$-$CH_2$- | 76 Lit [14] | 4.120 | - |
| 10 | H | H |  | H | H | - | 81 | 2.860 | - |
| 11 | CN | H | CN | CN | H | | 25[g] | 0.304 | - |
| 12 | NH$[CH_2]_2$-OH | H | H | H | NH$[CH_2]_2$-OH | - | - | 0.693 | 14 |
| 13 | NH$[CH_2]_2$-OH | H | H | H | H | - | - | -0.198 | 44.7 |
| 14 Lit [15] |  | H | H | H | H | - | - | 2.906 | 47.1 |

[a] Reference ([12], [15])for experimental data of respective compound.
[b] Reference ([14]) for experimental data of respective carboxylic acid.
[c] log *P* value of nitrile-containing compounds calculated using program HINT [16], the values in brackets were determined from $^{14}$C-labelled compounds [12] according to Ref. [17] and indicate positive deviation of the calculated values due to inadequate consideration of the steric features of di-*tert*-butylated phenols.
[d] Differential volume obtained by calculation differences (integration of exceeding volumes) to the model of the binding pocket (Fig. 1).
[e] Yield salicylic acid.
[f] Dependent on conformation.
[g] Yield 1,2,4,5-tetrabenzoic acid.

Table 2
Enzymatic hydrolysis of nitriles

| | | Yield (%) Lit[a] | log P[b] | Volume[c] (Å$^3$) | Volume[d] (Å$^3$) |
|---|---|---|---|---|---|
| 15 |  | 51 | 2.010 | - | 112 |
| 16 |  | 47 | 1.452 | - | 140 |
| 17 rac. |  | 38[e] Lit [18] | 1.189 | - | 171 |
| 18 |  | 35 | 2.720 | - | 128 |
| 19 |  | - | 3.880 | 10.4 | 167 |
| 20 |  | 32 Lit [19] | 3.880 | - | 167 |
| 21 rac. |  | - | 2.212 | 22.1 | 216 |
| 22 rac. |  | - | 3.594 | 74.7 | 246 |
| 23 |  | - | -2.476 | 34.9 | 248 |
| 24[f] |  | - | 0.901 | 23 | 171 |
| 25 |  | - | 3.534 | 15.4 | 156 |

[a] Reference for experimental data of respective carboxylic acid, **17**: [18], **20**: [19].
[b,c] See Table 1, footnotes c, d.
[d] Total volume (nitrile containing compound).
[e] Characterized as methyl ester [18].
[f] Mixture of D/L and *meso*.

the Novo nitrilase preparation. For optimal interpretation of structural features and to facilitate the mapping of steric constraints, conformationally restricted nitriles carrying a carbocycle, heterocycle or an aromatic system directly linked to the nitrile functionality were preferentially examined. As shown in the tables, all 13 substrates were transformed to the corresponding carboxylic acids and no amides were isolated. This indicates that observed restrictions for substrate binding are valid for the nitrilase and the hydratase enzyme but do not allow any conclusion about the amidase activity [1]. A strong prevalence of carboxylic acid products is not uncommon [1], the absence of any amide found in our experiments may be explained by the relatively long incubation time generally used (7 days) leading to the hydrolysis of possible amide intermediates. In some cases, very small quantities of byproducts were observed by TLC; however, these compounds could not be characterized further.

To map constraints on the basis of substrate profiles, structures were generated, optimized by force-field and subsequent AM1 calculations and the molecular volumes approximated by calculation of each molecules Van der Waals volume. The structures of all compounds were then overlaid by fitting the nitrile functions and positioned as described in the Experimental section. The combined volumes of all substrates thus describe the minimal dimensions of the binding pocket (Fig. 1).

Several reasons made us focus on the region close to the CN group. It is reasonable that hydrolysis of a compound requires access to catalytically active residues of the function to be converted. Consequently, productive binding should be predominantly sensitive to the steric impact of substituents close to the nitrile function. Moreover, with large molecules, catalysis

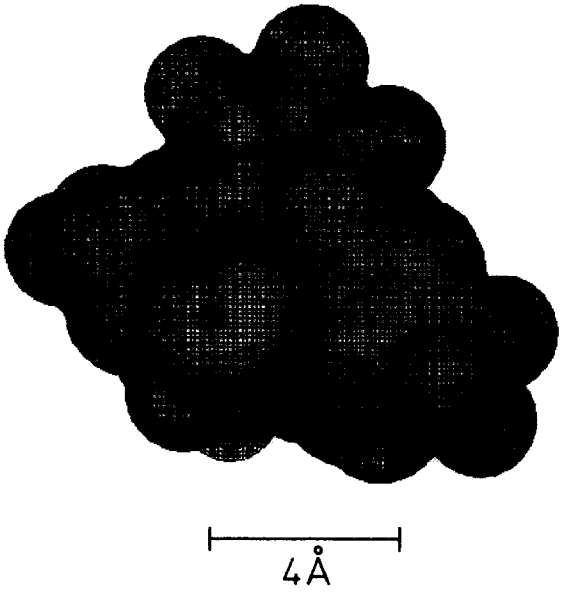



Fig. 1. Active site model for SP 409 derived from volumes of all substrates (Tables 1 and 2) three-dimensional representation up to 4 Å distance from the CN-nitrogen atom in both directions along the 'CN axis', top perspective view; '●' labels the position of the nitrile nitrogen.

may proceed with one part of the substrate filling the binding pocket while another part is placed at the outer surface and exposed to the solute.

To visualize the boundaries of the substrate-based model of the binding pocket (Fig. 1) in comparison to unprocessed compounds, volume differences of aligned hydrolyzed and unprocessed compounds were examined by slicing the overlays at increasing distance from the CN nitrogen atom (Fig. 2a, b). The crossing envelope surface lines show that substrates differ in molecular dimensions from nitriles not processed at any displayed level. When individual envelope surface-cuts of unhydrolyzed compounds were analyzed up to an (arbitrarily chosen) 4 Å limit from the nitrile nitrogen in respect to enzymatic turnover, the susceptibility to hydrolysis was found to be linked to the respective molecular dimensions, or else, nitriles not hydrolyzed exceeded the dimensions defined by all substrates (not shown). Compounds not processed by the enzyme thus are

[1] Since active site properties of the nitrilase and hydratase enzyme cannot be distinguished, both enzymatic activities are designated by the term 'nitrilase' in the text that follows.

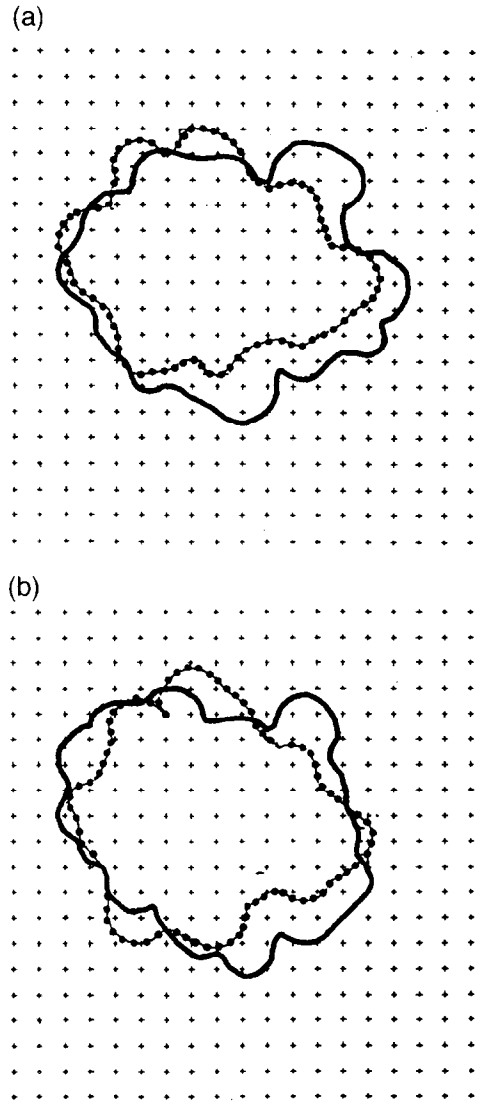



Fig. 2. Surface envelopes of aligned structures of compounds **1–25** [(—) confining all 13 superpositioned substrates, (· · ·) all 12 compounds not processed], generated by cutting the volumes at varying distance from the nitrogen atom of the nitrile-function, top perspective view along the 'N–C axis', in 'N–C' direction: (a) 2 Å, (b) 3 Å; grid overlay (20 Å × 20 Å, 1 Å spacing); the differential volume of those parts of each unhydrolyzed compound exceeding the surface of the substrate-based model is specified in the tables.

characterized by envelopes exceeding those of the model at various levels. For example, linear linkage of the third phenyl ring in the anthracene nitrile **19** impairs accommodation of the substrate in the binding pocket, most likely due to steric hindrance while the phenanthrene derivative **20** is hydrolyzed. The common surface envelope of all substrate nitriles, displayed in Fig. 1, thus describes the minimal dimensions of an essential part of the binding pocket confining a volume of approximately 227.9 $Å^3$.

As expected, a comparison of total molecular volumes e.g. of compounds **17**, **19**, **20** and **24** (Table 2) revealed that this value is an inappropriate parameter to assess the probability of enzymatic hydrolysis. While the anthracene and the phenanthrene nitrile **19** and **20** as well as compounds **17** and **24** exhibited the same value (167 $Å^3$ and 171 $Å^3$, respectively), only **20** and **17** were hydrolyzed. Also nitrile **25**, a compound with a volume 15 $Å^3$ smaller than substrate **17** was not hydrolyzed. Hence, enzymatic hydrolysis seems to be determined by the molecular shape, not by the total Van der Waals volume.

In contrast, when differential volumes of parts of aligned structures were calculated, data were found to be of potential predictive value. Volume difference calculations from partial structures consistently gave positive values (due to residues arranged outside the modelled binding pocket) only for those compounds not processed by the nitrilase (see Tables 1 and 2). Calculation of partial volumes of a distinct nitrile containing compound and subsequent comparison to our substrate-based model therefore is likely to give a forecast for enzymatic nitrile conversion with some reliability.

Some ambiguity however resulted from conformational flexibility, especially when compounds carrying 'flexible' spacer groups like the nitriles **5** and **6** were analyzed. To estimate the limitations of our model in this respect, compounds **5** and **6** were subjected to a conformational analysis, and low-energy conformations were then placed in the active site model. The plurality of the conformations differing less than 4 kcal $mol^{-1}$ (of both compounds) gave 'positive differential volumes' (not shown), indicating no fit to the model and consequently, in agreement with the experiment, no hydrolysis.

However, the value of energy calculations of isolated compounds is limited as the actual active-site conformation may significantly differ due to specific interactions with the enzyme. Positioning of flexible compounds however is a general problem, especially when no information about specific substrate–protein interactions is available.

The differential volumes of conformationally flexible compounds thus vary along with conformational changes, making predictions less reliable. It cannot be excluded however, that for compounds with very bulky moieties like the 2,5-di-*tert*-butyl-phenyl group (**4–6**), hydrolysis is hampered by an additional 'bottleneck' (not described by our model), precluding access to the active site.

To further analyze the impact of steric features, all 25 tested structures were subjected to a SYBYL [20] CoMFA [21] analysis. Steric fields were probed using an uncharged C $sp^3$ atom and interaction energies with all atoms were calculated. The 'activity' was expressed assigning the value $-1$ to all 'inactive' (not hydrolyzed) compounds and the value 1 to all substrates corresponding to a binary representation of 'activity' by a very roughly digitized distribution. This protocol was based on the rationalization that field analysis does not require linear function [22]. We therefore generated a virtual function $F(x) = -1$ for 'inactive' and $F(x) = 1$ for 'active' compounds in which $x$ comprises all the parameters (descriptors) of the molecule. The field values were subjected to CoMFA with the numerical activity to be predicted. All columns with a minimum sigma value of 2 were deleted and the remaining 833 columns were analysed using the standard PLS algorithm, predicting the activity within a numerical range from $-1.3$ to 1.3. Nitrile hydrolysis ('activity') then is reflected by values $> 0$ whereas values $< 0$ indicate lack of enzymatic hydrolysis. Based on these criteria, there were only two compounds (**7** and **19**) which were predicted incorrectly. The resulting predicted vs. actual 'activity' is depicted in Fig. 3 (cross validation (leave-one-out)). Each structure is represented by one data-point, actual values being either $-1$ or 1, predicted values being in the range of $-1.3$ to 1.3. The diagram is divided into quar-

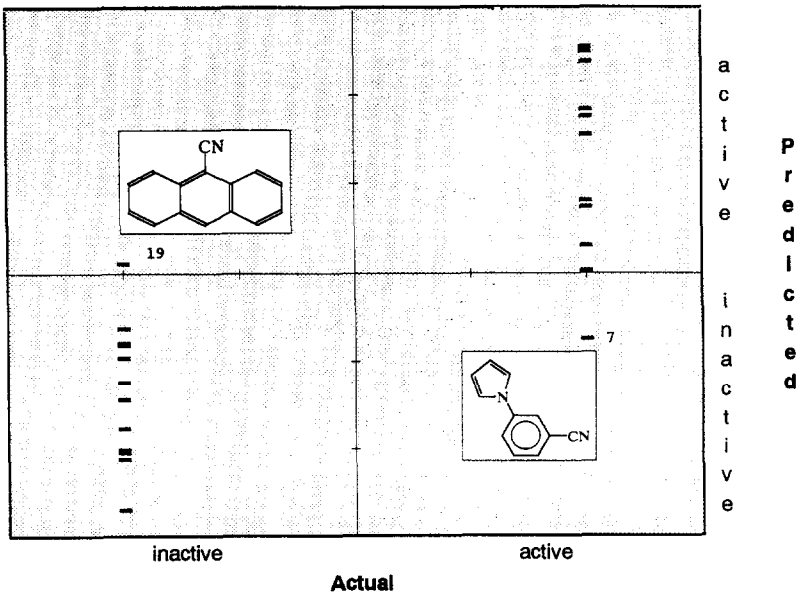



Fig. 3. Graphic representation of the results of a steric field analysis (CoMFA) involving all 25 nitrile-containing compounds; each data-point designates actual or predicted 'activity' in terms of enzymatic hydrolysis; for details see Results and discussion section and Experimental section.

ters confined by the 0/0-axes and can be interpreted as follows. Location of a data-point (a compound) within the first and third quadrant reflects correct prediction while falsely predicted compounds are found in the second and fourth quadrant. As already mentioned above, our method failed for only the two nitriles **7** and **19**. Moreover, the result of the differential volume calculation as for the anthracene nitrile **19** (Table 2) suggests that this compound represents a border-line case giving a value of only 10.4 Å$^3$. A correlation coefficient ($r^2$) of approximately 0.7 indicates that the partial influence of the steric descriptor of this single-variable CoMFA model explains 70% of the variance in the data ($r^2_{fit} = 0.696$, $n = 25$, $s_{fit} = 0.574$, $r^2_{cross} = 0.45$). In this context it must be mentioned that the punctual representation of the steric fields is different to the total volume calculation method as 'caves' inside or on the surface of the molecules may cause variations of the interaction energies at the grid points. Also these 'caves' do not affect the binding of the molecules to the catalytic site. Therefore the 'volume method' is likely to give a better description of (parts of) the binding site than the grid built by the RMS values of the CoMFA grid points. Nevertheless the results of the CoMFA method are in agreement with those of the alternative method in terms of putting great emphasis on steric features affecting enzymatic turnover.

Besides steric hindrance, insolubility or exceeding hydrophilicity could possibly impair hydrolysis in some cases. To account for this possibility, log$P$ values were calculated [16] or determined experimentally [17] (Tables 1 and 2). All non-processed compounds, except **13** and **23** exhibit values within the range covered by substrates [(log$P$ = 0.304 (**11**) to 4.97 (**7**)]. The lack of hydrolysis observed for **13** and **23**, which both display 'positive differential volumes', is probably not due to exceeding hydrophilicity as complete hydrolysis of the 1,2,4,5-benzonitrile **11** also requires hydrolysis of polar carboxy-intermediates. In earlier studies, hydrolysis was observed for polar carbohydrate nitriles [8] although SP 409 failed to transform polar cyanocarboxylic acids [7]. Lipophilicity does not appear to be a parameter relevant to enzymatic hydrolysis of the above compounds, however, limitations imposed by other than steric factors cannot be ruled out.

In conclusion, we have been able to demonstrate that molecular modelling is also a useful tool to assess enzymatic hydrolysis of potential substrates by a very crude mixture of several enzymatic activities. This has been achieved by the generation of a hypothetical model of the 'active site', which, despite the possibility that it is likely to represent a mixture of the properties of several hydrolytic activities present in the preparation, still appears useful in predicting enzymatic conversion. We do not claim that steric hindrance is the sole cause preventing enzymatic conversion by nitrilase SP 409, however, in this study it could be identified as highly relevant to explain lack of nitrile hydrolysis. By means of differential volume calculation, an appropriate molecular volume has been established as a necessary, and with some probability a sufficient prerequisite for enzymatic conversion. In any case, lacking fit to the above model is highly likely to indicate that a distinct compound will not be processed by the SP 409 enzyme system. Consistently, a 70% contribution of steric fields for the explanation of variance was found in a CoMFA study confirming the impact of steric bulk on hydrolysis. In turn, conversion of untested compounds should be predictable (with some probability) on the basis of differential volume calculations implying comparison to the dimensions of the parent model, alternatively by molecular (steric) field analysis.

## 3. Experimental

### *3.1. General*

Melting points were determined on a Mettler automatic FP 61 and are uncorrected. Thin layer

chromatography (TLC) was performed on Polygram SIL G/$UV_{254}$ plates from Machery–Nagel. Proton magnetic resonance spectra were recorded on a Bruker WM-250 or WM 300 spectrometer in $CDCl_3$. Chemical shifts ($\delta$) are reported in ppm with TMS as internal standard, $J$ values are recorded in Hz. Mass spectrometry was carried out on a Varian MAT 311A.

Nitrilase *Rhodococcus sp.* (SP 409, an immobilized preparation derived from *Rhodococcus sp.* CH5) was obtained from Novo Nordisk (Bagsvaerd/Denmark). With the exception of 2,6-bis-(2-hydroxyethylamino)-benzonitrile **12**, 2-(2-hydroxyethylamino)-benzonitrile **13**, 2-benzoylamidobenzonitrile **14** and the nitriles **4–6**, the compounds tested were from commercial sources. The benzonitrile **14** was prepared from anthranilonitrile as described [13]. The di-*tert*-butylphenol containing nitriles **4–6** were prepared by published procedures [12].

### 3.2. Enzymatic hydrolysis

In a typical reaction, 50 mg Nitrilase SP 409 (hydratase activity, substrate proprionitrile 391: HPU $g^{-1}$, amidase activity, substrate propionamide: 159 APU $g^{-1}$ [8]) was suspended in 5 ml potassium phosphate buffer (100 mM (= 0.1 mol $dm^{-3}$), pH 7.4) at 25°C and stirred for 1 h. The respective nitriles were then suspended giving a final 'concentration' of 50 mM. The mixtures were shaken at 200 rpm at 25°C for 7 days, and the progress of the reaction was monitored by TLC. Work up started with the removal of the enzyme by filtration through a Celite pad, basification of the filtrate with sodium hydroxide (pH 9) and extraction with 5 ml of chloroform, then diethyl ether to recover any unreacted material. The aqueous layer was then acidified with hydrochloric acid (pH 1) and extracted 3 times with 5 ml of chloroform and diethyl ether to recover acidic products. The combined organic layer was evaporated, the product isolated, and the identity of all compounds was checked by TLC [hexane/acetic acid ester 2:1 (vol) as eluent], by determination of mps or by $^1$H-NMR, mass spectrometry or elemental analysis and data compared with authentic material or published values. Analytical data are provided for novel compounds, otherwise the respective reference is included. No attempts were made to assess the stereospecifity of the reaction.

### 3.3. 2,6-bis-(2-Hydroxyethylamino)-benzonitrile 12 and 2-(2-hydroxyethylamino)-benzonitrile 13

2-Fluoro- or 2,6-difluorobenzonitrile (5.19 mmol) was reacted in aminoethanol (5 ml, 83.2 mmol) for 3 days at 80°C. The reaction mixture was then poured into water (20 ml) and extracted 3 times with chloroform. Evaporation of the solvent afforded the crude product which was purified by silica gel chromatography (hexane/ethyl acetate 1:1 to chloroform/methanol 5:1, v/v as the eluent) to give 2,6-bis-(2-hydroxyethylamino)-benzonitrile **12**, 2-(2-hydroxyethylamino)-benzonitrile **13** respectively.

### 3.4. 2,6-bis-(2-Hydroxyethylamino)-benzonitrile 12

(895.4 mg, 78%), mp: 107°C (Found: C, 59.42; H, 6.70; N, 18.67. calc. ($C_{11}H_{15}N_3O_2$, 221.26) C, 59.71; H, 6.83; N, 18.99); $\delta_H$ (250 MHz; $CDCl_3$) 3.32 (4H, t, $-NH-CH_2-CH_2-$) 3.79 (4H, t, $-NH-CH_2-CH_2-$) 4.89 (2H, s, –NH–) 5.10 (2H, br. s, –OH) 5.98 (2H, d, Ph. H) 7.17 (1H, t, Ph. H); $m/z$ (electron impact 70 eV, 120°C) 221 ($M^+$, 66%), 190 (100), 172 (27), 146 (27), 131 (18) and 104 (17).

### 3.5. 2-(2-Hydroxyethylamino)-benzonitrile 13

(538.7 mg, 64%), mp 38°C (Found: C, 66.41; H, 6.15; N, 17.12. calc. ($C_9H_{10}N_2O$, 162.19) C, 66.65; H, 6.21; N, 17.27); $\delta_H$ (250 MHz; $CDCl_3$) 3.38 (2H, t, $-NH-CH_2-CH_2-$) 3.86 (2H, t, $-NH-CH_2-CH_2-$) 4.88 (1H, s, –NH–) 6.66 (1H, dd, Ph. H) 6.79 (1H, d, Ph. H) 7.32 (1H, dd, Ph. H) 7.39 (1H, d, Ph. H) 8.42 (H, br.

s, –OH); *m/z* (electron impact 70 eV, 44°C) 162 ($M^+$, 29%), 131 (100), 104 (19), 77 (18) and 43 (23).

### 3.6. Molecular modelling

Calculations were performed on a Silicon Graphics SG 4D 35 TG work-station. The structures were generated with the SYBYL 6.03 program package [21] and energy minimized with the TRIPOS force field using standard atom parameters, then subjected to AM1 calculations using MOPAC 5.0 [23] (Keywords '*MMOK PARASOK PREC DENSITY LOCAL VECT MULLIK PULAY AM1 PI BONDS GRAPH*'). All compounds were aligned manually, fitting the atoms of the nitrile function and the adjacent carbon atom; the C–CN axis was oriented along the internal *z* axis keeping the coordinates of these atoms fixed, aromatic compounds were aligned in a coplanar orientation, thus affording a minimum total Van der Waals volume of superimposed structures. Molecules not symmetric to the axis defined by NC–C($R_x$) were oriented in a way that bulky substituents or annelated rings were arranged in the same direction avoiding any high energy operation; Van der Waals volumes were calculated with the SYBYL VOLUME option. The partial model of the binding pocket was generated by cutting the minimal common volume of aligned substrates at a 4 Å distance form the CN nitrogen (90° to the linear extension of the C–CN axis in both directions). Differential volumes were computed by integral volume subtraction from the substrate-based model and each aligned compound using the MVOLUME option (grid spacing 0.1 Å). Positive values from differential calculations reflect volume areas outside the surface envelope of the model.

SYBYL CoMFA calculations were carried out using all 25 structures, minimized with the Tripos force field and aligned as described (nitrile function oriented in *z*-direction). The interaction energies of an uncharged C $sp^3$ carbon (probe) with all atoms of each molecule (target) was calculated within a 'region' of −4 to 4 Å in *z*-direction and −12 to 12 Å in both *x* and *y* direction, the nitrile nitrogen being placed in the center of the coordinate system (grid spacing 2 Å, totally 845 grid points, 'steric only', non-bonded cutoff 12 Å). Cross-validation parameters: epsilon: $1 \times 10^{-4}$, iteration: 500, scaling method: autoscale.

The conformational analysis of compound **6** was carried out with the SYSTEMATIC SEARCH option involving all rotatable bonds of the nitrile–aromatic hydrocarbon spacer: 30° increments, no electrostatics, trans conformation of the amide function, calculation in vacuo since no intramolecular hydrogen bridges were formed.

The cartesian coordinates of all superimposed compounds, a SYBYL contour map of the active site volume model and a SPL script are available upon request to perform the operations described above and to probe potential substrates.

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