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Large α-aminonitrilase activity screening of nitrilase superfamily members: access to conversion and enantiospecificity by LC-MS.

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1. Introduction.

Natural and non-natural α-aminoacids (α-AA) are key chiral building blocks for the synthesis of fine chemicals.[1] Common proteinogenic α-AA can be obtained directly from natural sources while others are mainly obtained by asymmetric synthetic routes often requiring non environmentally benign conditions.[2] In that context, biocatalysis appears to be a favorable method for chiral synthesis purpose.[3] Hydrolytic enzymes such as acylases, hydantoinases, carbamoylases have been largely used for resolution and dynamic kinetic resolution processes on protected form of racemic α-AA.[4] Starting from α-aminonitriles (α-AN), α-AA have been obtained by the enzyme duo nitrile hydratase and amidase [5-8] or by the sole action of a nitrilase. Example of asymmetric synthesis of α-AA through the combined action of three enzymes (nitrile hydratase, aminopeptidase and ACL racemase) has been recently published.[9]

Most examples reported the synthesis of chiral α-AA from α-AN via whole cells, only a few dealt with the production with isolated nitrilases. In their pioneer work, Choi *et al* reported the synthesis of (*S*)-phenylglycine by free cell extracts of *Asperigillus furmigatus*.[10] Few years later, various α-AN were found to be substrates of a nitrilase isolated from *Rhodococcus rhodochrous* PA-34 leading to (*S*)- or (*R*)-α-aminoacids.[11] However, even sounding promising, the biocatalytic synthesis of α-AA from α-AN with nitrilases was rarely exploited.[12-15]

We recently reported the creation of a large collection of nitrilases selected by sequence comparison.[16] In order to discover new α-aminonitrilases that catalyse the formation of chiral α-AA, we decided to extend this selection to better cover the whole biodiversity of the nitrilase superfamily. The enzymes were so also selected by shared protein domains (protein signature, Pfam, InterPro). We screened this larger collection of 588 enzymes as cell lysates against various α-AN. The six α-AN selected substrates (**1**-**6**) are precursors of natural and non-natural α-AA with diversified side chains (alkyl, polar, aromatic) (Figure 1). The HTS nitrilase assay is generally based on the ammonia detection liberated during the reaction.[17] With α-AN substrates, this assay is not feasible due to the loss of NH_3 in the reverse Strecker reaction (Scheme 1). Moreover, it doesn't give access to the enantiospecificity of

the reaction. Numerous methods have been developed to monitor the conversion rate and the stereochemical outcome of biocatalytic reactions. Among them, chiral HPLC fitted with UV detector is one of the most efficient and reliable methods, but it is mainly suitable for the detection of conjugated derivatives and require expensive and specific chiral column. In this study, we present a liquid chromatography-electrospray ionization tandem mass spectrometry (ESI-MS/MS) method for reliable measurement of six compounds. The LC approach uses achiral conventional C18 column with prior derivatization of the reaction mixture with a chiral UV chromophore. MS/MS detection is achieved by scanning through Multiple Reaction Monitoring (MRM)[18] events on a triple quadrupole instrument. MRM involves selecting for ions of a specified parent molecular weight (*m*/*z* ratio), fragmenting the parent ion at an optimal collision energy for producing particular daughter ions, and then quantifying the production of the ions of interest. This simple method affords a quick and reliable assay for nitrilase activity, giving access to both conversion rate and enantiospecificity.

From our activity screening, we obtained five nitrilases exhibiting an α-aminonitrilase activity. On the basis of their different catalytic capabilities, three were purified and further studied. From the six α-AN, only 2-aminovaleronitrile and 2-amino-2-phenylacetonitrile (norvaline and phenylglycine precursors, respectively) were found to be substrates. To extend the substrate scope, three 2-amino-2-phenylacetonitrile derivatives **7**-**9** (Figure 2), precursors of valuable synthons, were tested on discovered α-aminonitrilases and two of them were hydrolyzed into their corresponding α-AA by two different enzymes.

2. Material and methods.

2.1 Chemicals

All reagents were purchased from commercial sources and used without purification. Racemic 2-amino-2-phenylacetonitrile (**5**) was purchased from Sigma Aldrich. Racemic 2-amino-3-methylbutanenitrile (**1**) was prepared according to the procedure previously described by López-Serrano *et al*,[19] racemic aminophenylacetonitrile derivatives **7**, **8** and **9** following the one reported by Wang *et al*.[20] NMR spectra were recorded on a Bruker 300 MHz spectrometer (Evry University, France). Chemical shifts (expressed in ppm) of ^{1}H and ^{13}C NMR spectra were referenced to the solvent peaks δ H 3.31 and δ C 49.2 for MeOD-d_4, δ H 2.50 and δ C 39.5 for DMSO-d*6*. Thin-layer chromatography was performed with aluminium-backed sheets with silica gel 60 F254 (Merck). Column chromatography was performed on a CombiFlash® Companion using RediSep® Rf or GraceResolv Silica cartridges.

2.2 Synthesis

*2.2.1 Synthesis of 2-aminopentanenitrile as hydrochloride salt (**2**)* (AN-norvaline)

To a mixture of butyraldehyde (1.25 ml, 13.9 mmol, 1 equiv.) and zinc iodide (177 mg, 0.56 mmol, 0.04 equiv.) cooled to 0°C was added trimethylsilyl cyanide (2.18 ml, 17.2 mmol, 1.25 equiv.). The reaction mixture was stirred for 15 min at room temperature then a 7M solution of ammonia in methanol (10.5 ml) was added. The resulting solution was stirred for 2h at 40°C in closed system and concentrated to dryness under reduced pressure. Diethylether (30 ml) was added to the residue and the supernatant was dried over magnesium sulfate,

filtered and concentrated to dryness under reduced pressure. A solution of hydrochloric acid 2M in diethylether (7.5 ml) was added to the residue and the resulting solution was stirred for 30 min at room temperature and then concentrated to dryness under reduced pressure to afford the desired compound **2** as a slight yellow solid; yield: 1.31 g (70 %). ^{1}H NMR (DMSO-d*6*, 300 MHz) δ 0,90 (t, J = 7.2Hz, 3H, H5), 1.43 (m, 2H, H4), 1.86 (m, 2H, H3), 4.54 (t, J = 7.6 Hz, 1H, H2); ^{13}C NMR (DMSO-d*6*, 75 MHz) δ 13.2 (C5), 18.1 (C4), 32.1 (C3), 39.5 (C2), 116.9 (C1) (see SI, Figure S1 and S2).

*2.2.2 Synthesis of 2,6-diaminohexanenitrile as dihydrochloride salt **(3)*** (AN-lysine)

See SI Scheme S1 for the synthetic scheme

Benzyl (5-amino-5-cyanopentyl)carbamate

To a solution of oxalyl chloride (814 µL, 9.62 mmol, 2.3 equiv.) in dried dichloromethane (25 ml) under an inert atmosphere was slowly added a solution of dimethylsulfoxyde (1.3 ml, 18.2 mmol, 4.3 equiv.) in dried dichloromethane (10 ml). The solution was stirred for 5 min at -70°C then a suspension of benzyl (5-hydroxypentyl)carbamate)[21] (1 g, 4.22 mmol, 1 equiv.) in dried dichloromethane (25 ml) was transferred *via* canula. The mixture was stirred for 25 min at -70°C then triethylamine (11.7 ml, 8.44 mmol, 20 equiv.) was slowly added. The reaction mixture was stirred for 20 min at -70°C and 15 min at room temperature and then quenched by addition to 600 ml of water. The aqueous phase was extracted with diethylether (3 x 100 ml) and the combined organic layers were dried over magnesium sulfate, filtered and concentrated to dryness under reduced pressure to obtain the aldehyde (1.1 g) used in the next step without purification.

To a suspension of potassium cyanide (249 mg, 3.83 mmol, 1 equiv.) in water (520 µL) were added ammonium chloride (203 mg, 3.83 mmol, 1 equiv.) and ammonium hydroxide 33% (1.32 ml; 20.3 mmol, 5.3 equiv.). The reaction mixture was stirred for 15 min at 0°C then a solution of the aldehyde (900 mg, 3.83 mmol, 1 equiv.) in dioxane (1.35 ml) was added dropwise over 20 min. The mixture was stirred for 20 h and then quenched by addition of water (10 ml) and diethylether (30 ml). The aqueous phase was acidified with HCl 1M (50 ml), washed with diethylether (3 x 20 ml), basified to pH = 12 with NaOH 1M (20 ml) and then extracted with dichloromethane (4 x 10 ml). The combined organic layers were dried over magnesium sulfate, filtered and concentrated to dryness under reduced pressure to obtain the protected aminonitrile; yield: 905 mg (91 %). ^{1}H NMR (MeOD-d_4, 300 MHz) δ 1.52 (m, 4H, H4, H5), 1.71 (m, 2H, H3), 3.14 (t, 2H, H6), 3.71 (t, 2H, H2), 5.06 (s, 2H, H8), 7.34 (m, 5H, H10-H12); ^{13}C NMR (MeOD-d_4, 75 MHz) δ 23.9 (C4), 30.4 (C5), 35.9 (C3), 41.5 (C6), 44.2 (C2), 67.4 (C8), 123.1 (C1), 128.8-129.6 (C10-C12), 138.6 (C9), 159.0 (C7) (see SI, Figure S3 and S4).

*2,6-diaminohexanenitrile as dihydrochloride salt (**3**)*

To a suspension of dried sodium iodide (1.2 g, 8.03 mmol, 7.5 equiv.) in dried acetonitrile (6 ml) under inert atmosphere was added trimethylsilyl chloride (680 µL, 5.35 mmol, 5 equiv.). The mixture was stirred for 45 min at room temperature then the amine (279 mg, 1.07 mmol, 1 equiv.) was added dropwise. The reaction mixture was stirred for 4 h at room temperature and then concentrated to dryness under reduced pressure. Water (10 ml) and dichloromethane (10 ml) were added to the obtained crude oil. The two layers were separated, the aqueous layer was washed with dichloromethane (7 x 10 ml), neutralized with a saturated solution of sodium hydrogen carbonate and then lyophilized. Purification of the

product by flash chromatography on silicagel (MeOH + 0.1 % NH_4OH) afforded the 2,6-diaminohexanenitrile (112 mg). To a solution of this compound in methanol (10 ml) at 0°C was added dropwise a solution of hydrochloric acid 2M in diethylether (1.1 ml, 2.5 equiv.). The mixture was stirred for 15 min at room temperature and then concentrated to dryness under reduced pressure to obtain **3** as a very hygroscopic yellow solid; yield:157 mg, (75 %). ^{1}H NMR (MeOD-d_4, 300 MHz) δ 1.59-1.81 (m, 4H, H4-H5), 2.05 (m, 2H, H3), 2.99 (m, 2H, H6), 4.57 (t, J = 7.4 Hz, 1H, H2); ^{13}C NMR (MeOD-d_4, 75 MHz) δ 23.4 (C4), 27.7 (C5), 31.7 (C3), 40.3 (C6), 42.6 (C2), 116.8 (C1) (see SI, Figure S5 and S6). HRMS (ESI): m/z calcd. For $C_6H_{14}N_3$ $(M + H)^+$ 128.1182, found 128.1179.

*2.2.3 Synthesis of 4-amino-4-cyanobutanoic acid **(4)*** (AN-glutamate)

To a suspension of ethyl 4-amino-4-cyanobutanoate[22] (2.13 g, 7.29 mmol, 1 equiv.) in tetrahydrofuran (30 ml) was added a solution of hydrochloric acid 1M (7.7 ml). The reaction mixture was stirred for 30 min at room temperature then neutralized with a saturated solution of sodium hydrogen carbonate and concentrated to dryness under reduced pressure. Water (15 ml) and dichloromethane (15 ml) were added to the crude product. The two layers were separated, the aqueous layer was washed with dichloromethane (3 x 15 ml), acidified with a cooled solution of hydrochloric acid 5M until pH 5 and concentrated to a final volume of approximately 5 ml. The resulting suspension was centrifuged (12000 rpm, 4°C, 10 min) and the supernatant removed. Ethanol (10 ml) was added to the resulting solid, the suspension centrifuged (12000 rpm, 4°C, 10 min) and the supernatant removed. This procedure was repeated three times to obtain the desired aminonitrile **4** as a white powder; yield: 571 mg (61 %). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.82 (q, J = 7.4 Hz, 2H, H4), 2.35 (dt, J = 7.4 et 2,3 Hz , 2H, H3), 3.71 (t, J = 7.4 Hz, 1H, H2), 4.51 (s, 6H, NH_2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 29.9 (C4), 30.1 (C3), 42.1 (C2), 122.8 (C1), 173.7 (C5) (see SI, Figure S7 and S8). HRMS (ESI): m/z calcd. For $C_5H_9N_2O_2$ $(M + H)^+$ 129.0659, found 129.0658.

*2.2.3 Synthesis of 2-amino-3-(4-hydroxyphenyl)propanenitrile as hydrochloride salt (**6**)*

See SI Scheme S2 for the synthetic scheme

2-amino-3-(4-(benzyloxy)phenyl)propanenitrile

To a solution of 2-((4-benzyloxy)phenyl)acetaldehyde (200 mg, 0.89 mmol, 1 equiv.) (synthesized according to Frigerio *et al*.[23] from the corresponding alcohol[24]) in dichloromethane (4.5 ml) were added successively zinc iodide (1.1 mg, 0.035 mmol, 0.04 equiv.) and trimethylsilyl cyanide (122 µL, 0.97 mmol, 1.1 equiv.). The reaction mixture was stirred for 20 h at room temperature then concentrated to dryness under reduced pressure. The residue was dissolved in a solution of ammonia in methanol (7M, 9 ml) and the resulting solution was stirred for 4 h at room temperature. The residue obtained after concentration under reduced pressure was dissolved in a solution of hydrochloric acid 1M (5 ml). The aqueous layer was washed with diethyl ether (2 x 4 ml), basified to pH = 12 with a solution of sodium hydroxide 6M and then extracted with dichloromethane (3 x 5 ml). The organic layers were combined, washed with a solution of brine (5 ml), dried over magnesium sulfate, filtered and concentrated to dryness under reduced pressure to afford the desired protected aminonitrile as a yellow oil; yield: 214 mg (96 %).^{1}H NMR (MeOD-d_4, 300 MHz) δ 3.10-3.28 (m, 2H, H3), 4.69 (m, 1H, H2), 5.07 (s, 2H, H8), 7.01 (m, 2H, H5), 7.29-7.43 (m, H5 and H11-

H13); ^{13}C NMR (MeOD-d_4, 75 MHz) δ 37.1 (C3), 44.3 (C2), 71.1 (C8), 116.6 (C1), 126.3 (C6) 128.7-131.9 (C4-C5, C11-C13), 138.6 (C9), 160.3 (7) (see SI, Figure S9 and S10).

*2-amino-3-(4-hydroxyphenyl)propanenitrile as hydrochloride salt (**6**)*

To a suspension of protected aminonitrile (1.06 g, 4.93 mmol, 1 equiv.) in dichloromethane (90 ml) cooled to 0°C was added dropwise a 1M solution of boron tribromide in heptane (4.22 ml, 4.22 mmol, 0.85 equiv.). The reaction mixture was stirred for 2 h at room temperature and then concentrated to dryness under reduced pressure. Water (100 ml) and AcOEt (100 ml) were added to the residue, the two layers were separated and the aqueous layer was washed with AcOEt (4 x 50 ml), basified to pH = 11 with a solution of sodium hydroxide 2.5 M (10 ml) and then extracted with AcOEt (6 x 20 ml). The organic layers were combined, dried over magnesium sulfate, filtered and concentrated to dryness under reduced pressure. A solution of hydrochloric acid 2M in diethylether (20 ml) was added slowly to a precooled solution of the residue in AcOEt (2 ml). The mixture was stirred for 30 min at room temperature and then concentrated to dryness under reduced pressure. The crude product was triturated with AcOEt (5 x 10 ml) to afford the desired compound **6** as a brown powder; yield: 400 mg (41 %). ^{1}H NMR (MeOD-d_4, 300 MHz) δ 3.15 (m, 2H, H3), 4.65 (dd, J = 8.3 et 6.0 Hz, 2H, H1), 6.81 (m, 2H, H5), 7.19 (m, 2H, H6); ^{13}C NMR (MeOD-d_4, 75 MHz) δ 37.3 (C3), 44.4 (C1), 116.8 (C2), 117.1 (C5), 124.7 (C4), 131.9 (C6), 158.9 (C7) (see SI, Figure S11 and S12). HRMS (ESI): m/z calcd. For $C_9H_{11}N_2$ $(M + H)^+$ 163.0866, found 163.0800.

2.2 Protein selection

α-aminonitrilase activity is part of carbon-nitrogen hydrolysis activities, which are performing by enzymes belonging to the nitrilase superfamily[25] containing more than 10,000 sequences. To select candidate proteins in prokaryotic organisms, the strategy was based not only on the sequence identity with known nitrilases[16] but also on shared protein signatures from several resources: PFAM based on protein domains,[26] Gene 3D based on 3D structures[27] and Prosite based on sequence motifs.[28] Thus, sequences from UniprotKB,[29] having the PFAM identifier PF0079, the Gene 3D identifier 3.60.110.10 and the Prosite identifier PS50263, and coming from prokaryotic organisms were selected. We obtained 7,652 sequences which were divided into three main groups using different clustering methods (Kmeans, single linkage clustering): glutamine-dependent NAD(+) synthetase (1,131 sequences), apolipoprotein *N*-acyltransferase (1,279 sequences) and the rest of the nitrilase superfamily (5,242 sequences). Proteins from each set were compared by BLASTp[30] and from the alignment score, a distance was calculated. The sequences were clusterized with R hclust method and one sequence per cluster was selected in agreement with the availability of the corresponding DNA in the genomic Genoscope collection giving a total of 599 sequences (see SI Figure S13).

2.3 Cloning, candidate enzyme production and enzyme purification

All steps from primers purchase to biochemical assays were performed in 96-well microplates. The procedures used for gene cloning, protein production in *E. coli* BL21(DE3) plysE strains (Invitrogen), cell lysate preparations and enzyme purification of NIT28, NIT158 and NIT191 were carried out as previously described.[16] 425 candidate enzymes were obtained and added to the 163 of the previously published candidate nitrilase selection [16] giving a set of 588 candidate enzymes to be screened.

2.4 Enzyme assay and sample preparation for general activity screening

The activity screening was performed in 96-well microplates. The enzyme reactions were performed in a final volume of 100 µl containing 1 mM DTT, 3 µl cell lysate (0.05 to 0.1 mg/ml of total proteins), 10 mM substrate in 10% methanol at pH 7.3 (50 mM potassium phosphate buffer), at 30°C for 4h. The reactions were stopped with 3.5 µl of 25% TFA and 40 µl of each well of acidified reaction media were transferred to a 96-well daughter microplate and subjected to derivatization as described in paragraph 2.6. To minimize the number of microplates to be analyzed (42 microplates), two pools (pool I and pool II) of derivatized reaction media were generated according to MS detection criteria (see paragraph 2.7.1). 2 µl of each well of microplates containing derivatized reactions with substrates **2**, **3** and **6** (pool I) were combined in a 96-well daughter microplate and diluted with 194 µl of TFA 0.01% before LC-MS analyses (volume of injection: 10 µl). Similarly, another 96-well daughter microplate was generated for pool II (substrates **1**, **4** and **5**). Conversion rates and enantiomeric excess were determined by LC-MS analysis comparing derivatized substrate and product concentrations to standard curves of their racemic forms. The standards were prepared as described above replacing enzyme cell lysate by *E. coli* BL-21 blank cell lysate (see SI Figure S14).

2.5 Activity of the purified enzymes

*2.5.1 Activity of NIT191 on 2-aminovaleronitrile (**2**)*

10 mM of 2-aminovaleronitrile **2** were incubated at 30°C in 100 mM potassium phosphate buffer pH 7.3 with 0.1 mg of purified NIT191 in a final volume of 1.0 mL. An aliquot (100 µl) of the reaction mixture was quenched by addition of 1 µl of TFA and centrifugated (13000 rpm, 10 min) to pellet the precipitated protein. 8 µl of a 60 mM solution of L-alanine were added as internal standard to 40 µl of the supernatant and 40 µl of the resulting solution were subjected to (*S*)-NIFE derivatization as described in paragraph 2.6. The resulting solution was subjected to HPLC-UV analysis (injection 10 µl; conditions 1, *tR* [(*S*)-alanine] = 6.72 min, *tR* [(*S*)-norvaline] = 8.34 min, *tR* [(*R*)-norvaline] = 9.16 min). Conversion rates and enantiomeric excess were determined by comparing enzyme derivatized product concentration to standard curves of racemic products (standardized with L-alanine).

*2.5.2 Activity of NIT28 on 2-amino-2-phenylacetonitrile (**5**)*

10 mM of 2-amino-2-phenylacetonitrile (**5**) were incubated at 30°C in 100 mM potassium phosphate buffer pH 7.3 with 0.008 mg of purified NIT28 in a final volume of 1.0 mL. An aliquot (100 µl) of the reaction mixture was quenched after a specified period of time by addition of 1 µl of TFA, centrifugated (13000 rpm, 10 min) to pellet the precipitated protein and 5 µl of the supernatant were directly subjected to HPLC analysis (conditions 2, *tR* (PheGly) = 3.78 min, *tR* (PheGlyCN) = 7.55 min). Conversion rates were determined by comparing enzymatic product concentration to standard curves of synthesized racemic product. For determination of the enantiomeric excess, 50 µl of the supernatant were mixed with 20 µl of 1M K_2CO_3 solution and 100 µl of a solution of (*S*)-2-((5-fluoro-2,4-dinitrophenyl)propanamide) (FDAA) (6.4 mM in acetone). The resulting yellow solution was heated at 40°C for 1h30 min under stirring (1000 rpm) then quenched with HCl 1M (20 µl) ,

diluted with 100 µl of MeCN and filtered off before HPLC analysis (injection 10 µl; conditions 3, *tR* [(*S*)-PheGly] = 9.30 min, *tR* [(*R*)-PheGly] = 12.51 min).

*2.5.3 Screening of NIT28, NIT158 and NIT191 on 2-amino-2-phenylacetonitrile derivatives **7**, **8**, **9**.*

10 mM of aminonitrile were incubated at 30°C in 100 mM potassium phosphate buffer pH 7.3 with 0.005 mg of purified enzyme for 24h in a final volume of 50 µl. The reaction mixture was quenched by addition of 1 µl of TFA, centrifugated (13000 rpm, 10 min) to remove the protein and 40 µl of the supernatant mixed with 16 µl of 1M $NaHCO_3$ solution and 80 µl of a solution of FDAA (6.4 mM in acetone). The resulting yellow solution was heated at 40°C for 1h30 min under stirring (1000 rpm) then quenched with HCl 1M (16 µl), diluted with 170 µl of MeCN and filtered off before HPLC analysis (conditions 5, t*R* (**7b**-(*S*)) = 27.04 and t*R* (**7b**-(*R*)) 27.41 min; conditions 3, t*R* (**9b**-(*S*)) = 9.93 and t*R* (**9b**-(*R*)) = 12.52 min).

*2.5.4 Activity of NIT28 on 2-amino-2-(4-methoxyphenyl)acetonitrile (**9a**)*

Same protocol as described in paragraph 2.7.2 with 2-amino-2-(4-methoxyphenyl)acetonitrile (**9a**) as substrate and 0.11 mg of enzyme in a final volume of 1.0 mL. Analytical conditions: conditions 4 for determination of the conversion (*tR* (9) = 22.30 min) and conditions 3 for determination of the enantiomeric excess (t*R* (**9b**-(*S*)) = 9.93 and t*R* (**9b**-(*R*)) = 12.52 min).

2.6 Derivatization procedure with N-(4-Nitrophenoxycarbonyl)-L-phenylalanine 2-methoxyethyl ester ((S)-NIFE)

40 µl of the reaction mixture (acidified as described for deactivation of the enzyme) were basified to pH 7.5 with 3.5 µl of a 2.5 M K_2CO_3 stock solution. 16 µl of a 20 mg/ml solution of (*S*)-NIFE in acetonitrile were then added and the resulting yellow solutions were shaken for 20 min at room temperature. The derivatized media were subjected directly to HPLC-UV analysis or diluted (1/100) and pooled with 0.01 % TFA solution for LC-MS analysis to raise a pH around 6.5-7 (see paragraph 2.7).

2.7 Analytical methods

2.7.1 LC-MS analyses

LC/MS/MS analyses were carried out on a Dionex UltiMate 3000 RS LC system (Thermo Scientific Dionex Corporation, Sunnyvale, CA, USA) coupled to a Hybrid triple quadrupole-linear ion trap mass spectrometer (QTRAP 5500 from ABSciex, Toronto, Canada). MS/MS experiments were conducted in the triple quadripole mode. Preliminary MS experiments allowed pooling the derivatized enzymatic media in two groups before the LC-MS analysis, providing that pooled compounds do not have identical *m/z* ratio, leading to a shorten analysis time. (*S*)-NIFE derivatized α-AN **2**, **3** and **6**, synthetic precursors of nor-Val, Lys and Tyr respectively, were grouped in pool I, and (*S*)-NIFE derivatized α-AN **1**, **4** and **5**, synthetic precursors of Val, Glu and PheGly respectively, in pool II.
For HPLC separations before mass spectrometry analyses, an ACQUITY UPLC BEN C18 column (1.7 µm, 2.1 x 150 mm) from Waters (Milford, USA) at a flow rate of 400 µl / min was

used. The column was maintained at 50°C with a column heater. Mobile phases were (A) 0.1% TFA in water and (B) acetonitrile with the following gradient mode: ratio A/B 100/0 to 50/50 in 16 min and 50/50 to 0/100 in 5 min. The separation conditions were developed with standard mixtures of nitriles and corresponding aminoacids (stock solution at 10 mM in 50 mM potassium phosphate buffer pH 7.3 with 10% methanol diluted 100 times in water) derivatized as described in paragraph 2.6.
Mass spectrometry analyses were conducted in the positive ESI ion mode with the following parameters: ion source (IS) 5500 V, curtain gas (CUR) 20 a.u., temperature (TEM) 450°C, gas 1 (GS1) 45 a.u., gas 2 (GS2) 60 a.u., CAD – medium.
MS/MS experiments were carried out using MRM (multiple reaction monitoring) scan type allowing simultaneous monitoring of several compounds using multiple ion transitions and their subsequent quantitation in complex mixtures. For each compound of interest presented in the pools I and II the optimization of following MS parameters (declustering potential (DP), collision energy (CE) and cell exit potential (CXP)) was performed in order to establish the best intensity transitions. Two transitions for each compound were chosen to build the final MRM methods for pool I and pool II. In fact, each method includes a set of transitions for detection and quantitation of derivatized amino acids and corresponding amides and nitriles. (for more details see SI Table S1). Figure 3 illustrates separation and MS detection of derivatized α-aminonitriles and corresponding α-aminoacids in standard mixtures.

2.7.2 HPLC-UV analyses

For HPLC analyses, an Atlantis® T3 column (3 µm, 4.6 x 150 mm) from Waters (Milford, USA) at a flow rate of 1 ml/min was used. Mobile phases were (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile in the specified A/B ratio. Conditions 1: gradient mode (ratio A/B 70/30 to 50/50 in 20 min) and UV detection at λ = 210 nm. Conditions 2: ratio A/B 90/10 and diode array detection. Conditions 3: gradient mode (ratio A/B 65/35 to 45/55 in 20 min) and UV detection at λ=338 nm. Conditions 4: ratio A/B 99/1 and UV detection at λ=229 nm. Conditions 5: gradient mode (ratio A/B 80/20 to 50/50 in 20 min then isocratic mode 50/50) and UV detection at λ=338 nm.

3. Results and discussion

3.1 Choice of derivatization procedure

We set up a valuable high-throughput assay giving access to both conversion and enantiospecificity of the nitrilase reaction for a broad substrate scope. In place of Marfey's reagent (FDAA), commonly used for aminoacid analysis, which requires elevated temperature not compatible with α-aminonitrile stability, we chose the (*S*)-NIFE as the most suitable derivatization reagent. Already developed for aminoacid[31, 32] this carbamate revealed to be appropriate for α-aminonitrile derivatization as the reaction can occur at pH near neutrality (pH = 7.5) and room temperature. However, an exception was noted for 2-amino-2-phenylacetonitrile (**5**) and its corresponding derivatized α-aminoacid which are much more unstable.[12] Nevertheless, for this substrate, our procedure gave reliable qualitative information concerning the monitoring activity.

3.2 LCMS-screening method

We developed a procedure to perform a general screening of the candidate enzymes (588) against the various α-aminonitriles (6) in an "all against all" strategy, leading to a good quantification of both conversion rate and enantiomeric excess. Considering the large number of analyses to be performed, we set up a sensitive method with a compromise between quality and duration. Each targeted reaction was monitored through three parameters: retention time, abundance of two daughter ions produced by optimized fragmentation of the parent ion for each substrate and corresponding product (see paragraph 2.7.1 and SI Table S1). To minimize time required for analysis, the derivatized reactions were grouped into two pools. Two derivatized α-AN and α-AA (**1** and **2** and their corresponding α-AA Val and Nor-Val) which had the same parent mass (*m/z* 348.0 and 367.1 respectively) were consequently separated in different pools. After optimization, we were able to test 3528 reactions. As shown in Figure 3, baseline resolution was not perfectly achieved in all cases. Nevertheless, the aim of the screening was first to detect nitrilase activity and secondly to discriminate rapidly high enantiospecific nitrilases from very poor ones. This goal was attained in these conditions. The stereochemical configuration of produced α-aminoacids was assigned by comparison with enantiopure derivatized standards (*S*) and (*R*)-aminoacid LCMS chromatograms. The method provides high sensitivity as the smallest conversion found (5%, vide infra) gave a signal significantly greater than the background (see SI Table S2 for peak area data). Keeping in mind that the stereospecificity is a critical parameter, this screening method allows to directly assessing the viability of a nitrilase in a biocatalytic process. It offers a significant advantage over existing methods that just give qualitative information of conversion, e.g. spectrophotometry,[33, 34] microorganism cultures with nitriles as the sole source of nitrogen[11, 35, 36]. In these reported methods, only positive hits are then studied by chiral HPLC to validate or discredit them, thus consuming time and using expensive columns.[37]

3.3 High-throughput screening results

The candidate enzymes were screened under standard conditions (see paragraph 2.4). The results are summarized in Table 1. Out of the very large number of candidate nitrilases assayed, only five exhibited an α-aminonitrilase activity and only compounds **2** and **5** were hydrolyzed into their corresponding α-AA (Figure 4). Peak areas and conversions deduced are detailed in SI Table S2. These compounds were already known to be substrates of few nitrilases, giving the corresponding acids with good stereopreference for the (*S*)-norvaline (NitA from *Rhodococcus rhodochrous* PA-34)[11] or (*R*)-phenylglycine (Nit5086 from Diversa,[12] nitrilase from *Pseudomonas fluorescens* DSM7155 and NIT-106 from Codexis[37]). Nevertheless the conversion of α-AN **5** was obtained on whole cells[5, 10, 35, 36, 38], i.e. without knowing which enzyme catalyses the reaction, nitrilase alone or nitrile hydratase / amidase couple. No candidate was found for α-aminonitriles bearing basic (2,6-diaminohexanenitrile (**3**)), acidic (4-amino-4-cyanobutanoic acid (**4**)), benzylic (2-amino-3-(4-hydroxyphenyl)propanenitrile (**6**)) groups or ramified alkane chain (2-amino-3-methylbutanenitrile (**1**)). It is worthy to note that for the six substrates, none amide formation was observed. This undesired by-product is often a brake for the development of the biocatalytic nitrile hydrolysis. Therefore, our new enzymes are suitable candidates for hydrolysis of 2-aminovaleronitrile (**2**) and 2-amino-2-phenylacetonitrile (**5**). From our results,

one nitrilase (NIT191, Table 1 entry 1) exhibited a very good activity towards 2-aminovaleronitrile (**2**), together with a very high ee for the (S)-enantiomer, while this nitrilase plus four others (NIT09, NIT28, NIT29, NIT158) were found to be active towards 2-amino-2-phenylacetonitrile (**5**) (Table 1, entries 2-6). The (*R*) enantiomer of this α-aminoacid is of commercial interest as a raw material for cephalosporin synthesis.[39] Interestingly, four nitrilases were active towards the (*R*) α-aminonitrile enantiomer, the fifth one, NIT191, being active towards the (*S*) enantiomer although with a low specificity (entry 6). It is worthy to note that NIT191 was the only enzyme active towards both substrates. This enzyme, from *Burkholderia xenovorans* LB400, was already described[16, 40] as a nitrilase but α-AN were not yet reported as substrates. The determination of sequence identity between our five nitrilases and already published nitrilases[12, 41-44] catalysing the formation of (*S*)-norvaline and (*R*)-phenylglycine showed that enzymes annotated as nitrilases (NIT28 (A5V4Y8), NIT158 (A5VE02) and NIT191 (Q13VV3)) have a moderate (50%) to quite significant (75%) sequence identity (Table 2). The two others hits (NIT09 (B1MWQ8) and NIT29 (Q9Z5U5)), annotated as predicted amidohydrolase and hydrolase respectively, exhibit very low sequence identities with known ones (<30%, Table 2).

NIT191, together with NIT09, NIT28 and NIT158, has already been found in our laboratory to hydrolyse a broad range of substrates including α-hydroxynitriles. This illustrates the promiscuity found in the nitrilase family. These results suggest also that α-aminonitrilases are probably only very weakly present in nature as only a few were depicted, in these study and others, despite the different implemented protein selection methodologies covering the whole prokaryotic biodiversity.

3.4 Enantiospecificy profiles of selected hits with purified enzymes

NIT28, NIT158 and NIT191, exhibiting promising results according to conversion and enantiospecificity on cell lysates, were purified for further studies. One representative for both substrate **2** and **5** were chosen to check the reliability of our method as compared to existing ones. Conversion rates and enantiomeric excess over time for NIT191 from *Burkholderia xenovorans* on aliphatic aminonitrile **2** and NIT28 from *Sphingomonas wittichii* on aromatic aminonitrile are presented below.

The conversion of 2-aminovaleronitrile (**2**) by NIT191 and the enantiomeric excess of the obtained norvaline were monitored over 24 hours by HPLC-UV after (*S*)-NIFE derivatization (Figure 5). NIT191 appeared to be very stereospecific for the (*S*) enantiomer (*ee* 96%) as only 2% of derivatized (*R*)-norvaline can be detected after 24h and the final conversion attained 52% (RHEA: 40780). During the LCMS- screening, the deductive conversion from calibration curve was 53% and the corresponding enantiomeric excess 94% ee (entry 1, Table 1). These screening results are in very good accordance with the ones obtained with the purified NIT191. This high stereospecificity makes this enzyme a very good candidate for the formation of (*S*)-norvaline from the corresponding α-aminonitrile. To our knowledge, this result is the best reported for this biocatalytic transformation. The only other described example was the nitrilase from *Rhodococcus rhodochrous*, also (*S*) stereospecific but with a lower stereopreference.[11]

For the hydrolysis of 2-amino-2-phenylacetonitrile (**5**), the conversion was monitored by HPLC (conditions 2) without any derivatization process as phenylglycine presents a sufficient UV response to enable its direct UV-quantitation. The determination of the enantiomeric excess was deduced after chiral derivatization with the Marfey's reagent. Evolution of

conversion and enantiomeric excess over time are presented in Figure 6. NIT28 presented a high stereopreference for the (*R*) enantiomer but not total as 8% of the (*S*)-phenylglycine (*ee* 84%) was detected by HPLC-UV for a conversion of 49% (RHEA: 40776). Thus, this enzyme was found active towards this substrate as it was the case in the activity screening, but with a relative good enantiospecificity. That point is in discrepancy with the one obtained during the screening where NIT28 appeared very poorly stereospecific (entry 6, Table 1). In this particular case, our screening monitoring assay gave inconsistent results concerning enantiomeric excess, mainly due to medium instability during the acidic quench and weak re-basification to pH 7.5 for (*S*)-NIFE derivatization. This observation was not made for the others substrates, making our screening method still relevant for all the others tested α-aminonitriles. For this transformation, NIT28 appeared to be an alternative to the nitrilase 5086 from Diversa,[12] however with a slight lower enantiospecificity. Its good relative activity at temperature around 50°C on other substrates[16] makes it a potential promising stable biocatalyst. Thermal and pH stabilities / activities are under investigations in the laboratory. With the aim of broadening the substrate scope, the three purified enzymes were assayed on aminophenylacetonitrile derivatives **7a**-**9a** (Figure 2), precursors of valuable synthons. Diphenol **7a** and cyano compound **8a** are respectively direct precursors of 3,5-DiHydroxyPhenylGlycine **7b** (3,5-DHPG) and 4-MethylCarboxyPhenylGlycine (4-MCPG), two non-natural very expensive α-aminoacids with pharmacological interest in the treatment of Alzeihmer disease.[45, 46] The α-AA **9b** obtained by hydrolysis of α-AN **9a** is an intermediate in the synthesis of various active compounds. The results are summarized in Table 3. Unexpectedly, despite its structural similarity with substrate **5**, no activity was detected on derivative **8a**. NIT28 and NIT158 were found to be active towards both derivatives **7a** (entries 1-2) and **9a** (entries 5-6) giving predominantly the corresponding (*R*)-aminoacids; NIT191 gave a good conversion of the methoxy-derivative **9a** but with (*S*)-stereopreference (entry 7) as already depicted for **5**. NIT28, exhibiting the best enantiospecificity and activity towards α-aminonitrile **9a** was further studied (Figure 7). A satisfying enantiospecificity for the (*R*)-stereoisomer was observed as only 4.5% of the other enantiomer could be detected after a conversion of 26% over 20 h. However this enzyme was found less active on this methoxy derivative **9a** than on the former 2-amino-2-phenylacetonitrile (**5**).

4. Conclusion.

The high-throughput screening based on LC-MS analysis of derivatized substrates and compounds is a very efficient method to identify new enantiospecific activities leading to α-aminoacids. In our work, this method was applied to the identification of enzymes able to convert α-aminonitriles into their corresponding α-aminoacids. Five hundred eighty eight candidate enzymes representative of the prokaryotic biodiversity were selected following two criteria, their sequence identity with a set of known nitrilases or their phylogenetic position among the nitrilase superfamily. Despite the high number of candidate enzymes screened against a representative substrate set (alkyl, polar, aromatic), only five enzymes exhibiting an α-aminonitrilase activity were depicted. Interestingly, no nitrile hydratase activity was observed. Only a few α-aminonitrilases were already known and our large screening allows us to conclude that, probably, this activity is very rare among the nitrilase superfamily. Moreover, the substrate range was found to be very narrow with four α-aminonitriles

hydrolyzed by the enzymes of this study, 2-aminovaleronitrile, 2-amino-2-phenylacetonitrile and two derivatives thereof. Consequently the general access of non-natural α-aminoacids from α-aminonitrile precursors by biocatalytic hydrolysis with nitrilases is questioned. Nevertheless, the new α-aminonitrilases displayed interesting activities suggesting the potential use of these enzymes in biocatalytic synthesis of (*S*)-norvaline and chiral phenylglycine derivatives.

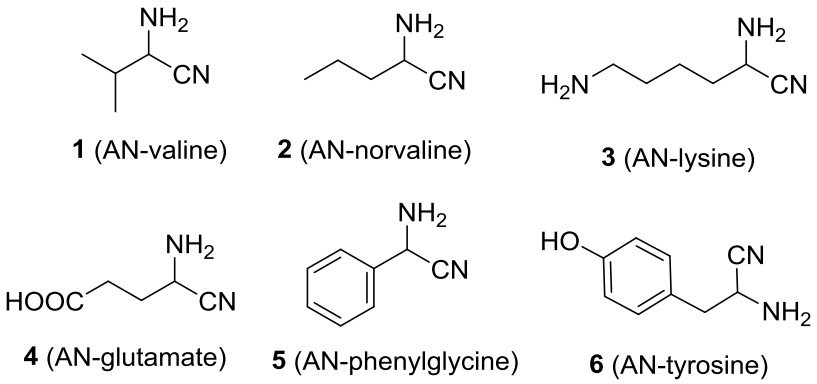
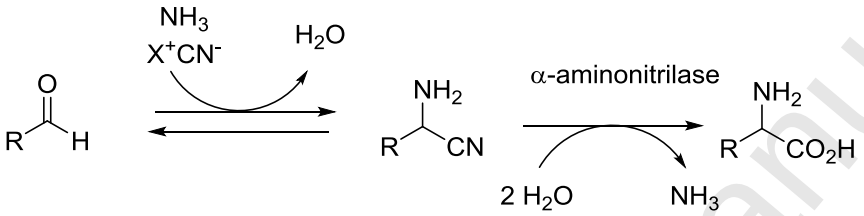


Fig. 1. α-aminonitrile structures.



Scheme 1. NH_3 formation in the nitrilase reaction with α-aminonitrile.

Table 1. Activity screening results: conversion and enantiomeric excess

Entry	NIT	Uniprot KB number	organism	substrate	conversion (%)[a]	ee (%)[a]
1	191	Q13VV3	*Burkholderia xenovorans*	**2**	53	94 (*S*)
2	09	B1MWQ8	*Leuconostoc citreum*	**5**	7	95 (*R*)
3	28	A5V4Y8	*Sphingomonas wittichii*	**5**	63	9 (*R*)
4	29	Q9Z5U5	*Zymomonas mobilis*	**5**	5	95 (*R*)
5	158	A5VE02	*Sphingomonas wittichii*	**5**	11	95 (*R*)
6	191	Q13VV3	*Burkholderia xenovorans*	**5**	64	≤5 (*S*)

See paragraph 2.4 for experimental details.
No conversion detected for substrates **1**, **3**, **4** and **6**.
[a] Determined by LC-MS after reaction medium derivatization by (*S*)-NIFE (see paragraph 2.6).

Table 2. Activity screening results: α-aminonitrilase hits and percent sequence identity with published nitrilases catalysing the formation of (*S*)-norvaline and (*R*)-phenylglycine

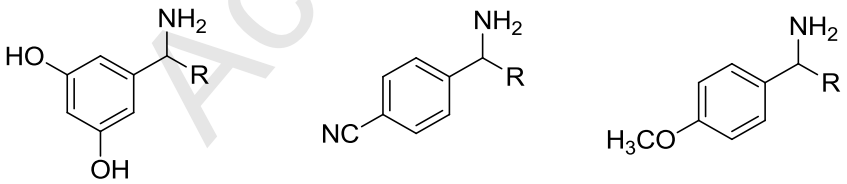
	(*R*)-phenylglycine producer				(*S*)-norvaline producer
	NIT09 (B1MWQ8)	NIT28 (A5V4Y8)	NIT29 (Q9Z5U5)	NIT158 (A5VE02)	NIT191 (Q13VV3)
K4U7B1 *(R. rhodochrous)*					50
Nit 5086 Diversa/ Q6RWK5 (Uncultured organism)	<30	61	<30	42	65
Q5EG61 *(P. fluorescens)*	<30	59	<30	48	75
NIT106 Codexis/Mutant of Q89GE3 (*B.diazoefficiens*)	<30	59	<30	46	60

The determination of optimal alignment and percent sequence identity was performed using the BLAST software publicly available through the National Center for Biotechnology Information website. Indicated values are the percent sequence identity.
For phylogenetic tree of these sequences, see SI Figure S15

Table 3. Activity results on derivatives **7a**-**9a**: conversion and enantiomeric excess

Entry	substrate	NIT	conversion (%)[a]	ee (%)[a]
1	**7a**	28	2	96 (*R*)
2	**7a**	158	1	74 (*R*)
3	**7a**	191	2	>99(*S*)
4	**8a**	28,158,191	*no product detection*	
5	**9a**	28	51	84 (*R*)
6	**9a**	158	93	42 (*R*)
7	**9a**	191	64	34 (*S*)

[a] see section 2.5.3 for experimental details



7a R = CN
7b R = CO_2H

8a R = CN
8b R = CO_2H

9a R = CN
9b R = CO_2H

Fig. 2. 2-amino-2-phenylacetonitrile derivatives

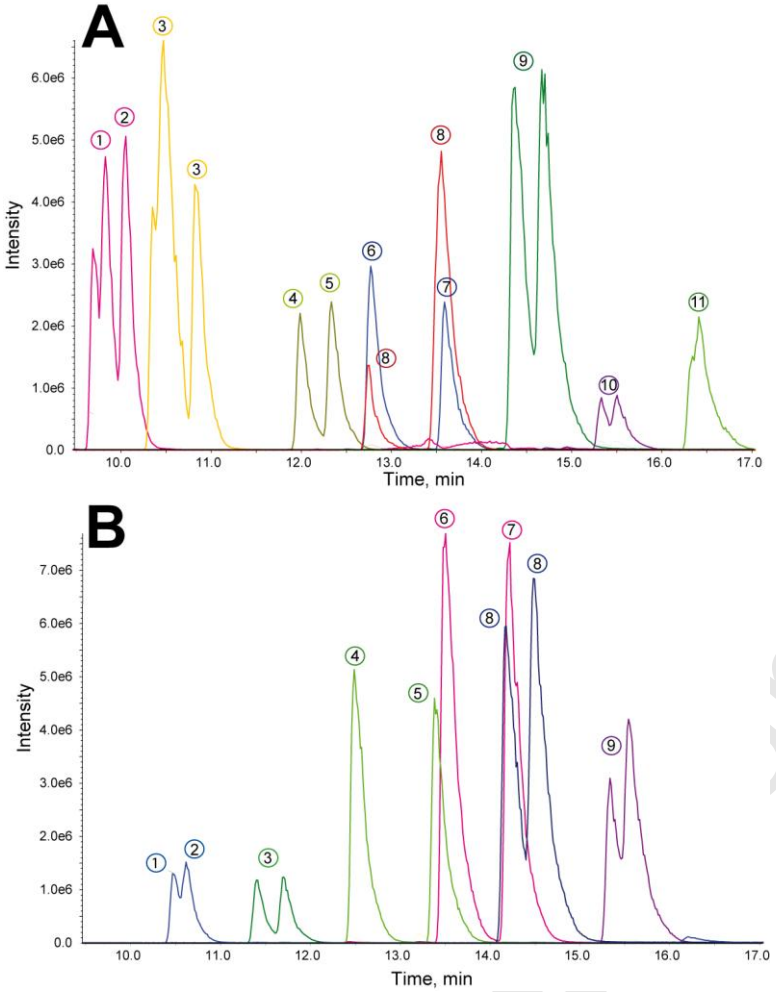
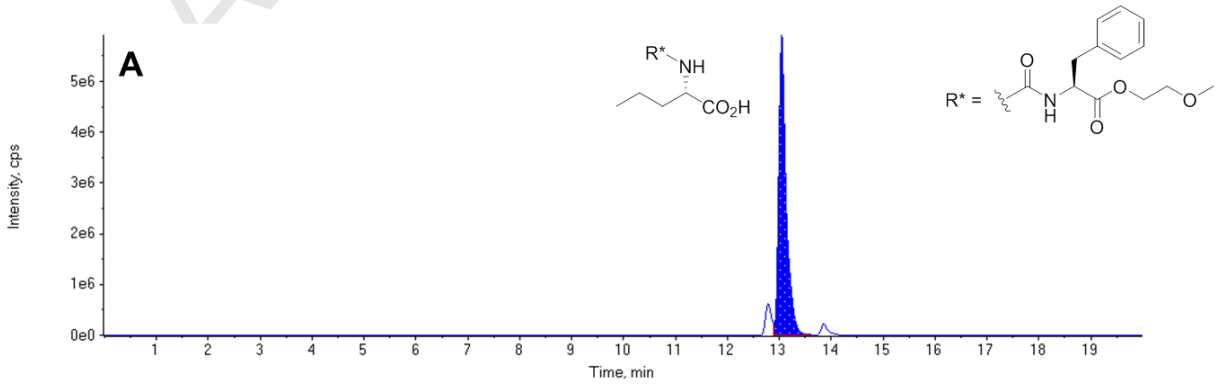


Fig. 3. LC-MS analysis of standards (*S*)-NIFE derivatized α-aminonitriles and corresponding α-amino acids. **A**: pool I. 1, (*S*)-lysine; 2, (*R*)-lysine; 3, AN-lysine; 4, (*S*)-tyrosine; 5, (*R*)-tyrosine; 6, (*S*)-norvaline; 7, (*R*)-norvaline; 8, AN-tyrosine; 9, AN-norvaline; 10, (*S*) and (*R*) doubly derivatized lysine; 11, AN-lysine. **B**: pool II. 1, (*S*)-glutamate; 2, (*R*)-glutamate; 3, AN-glutamate; 4, (*S*)-valine; 5, (*R*)-valine; 6, (*S*)-phenylglycine; 7, (*R*)-phenylglycine; 8, AN-valine; 9, AN-phenylglycine. For clarity, a single MRM transition is represented.



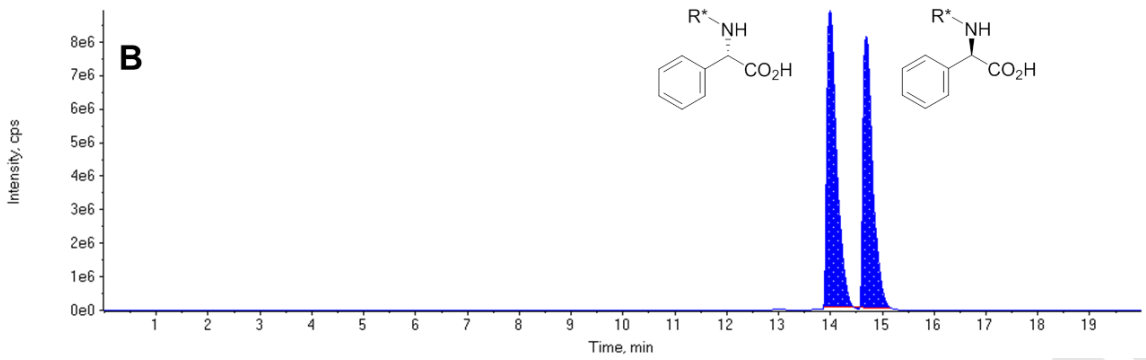


Fig. 4. **A**. MRM analysis of derivatized reaction medium NIT191 on pool I: detection of (*S*)-norvaline (94% ee) **B**. MRM analysis of derivatized reaction medium NIT28 on pool II: detection of (*R*)-Phenylglycine (9% ee).

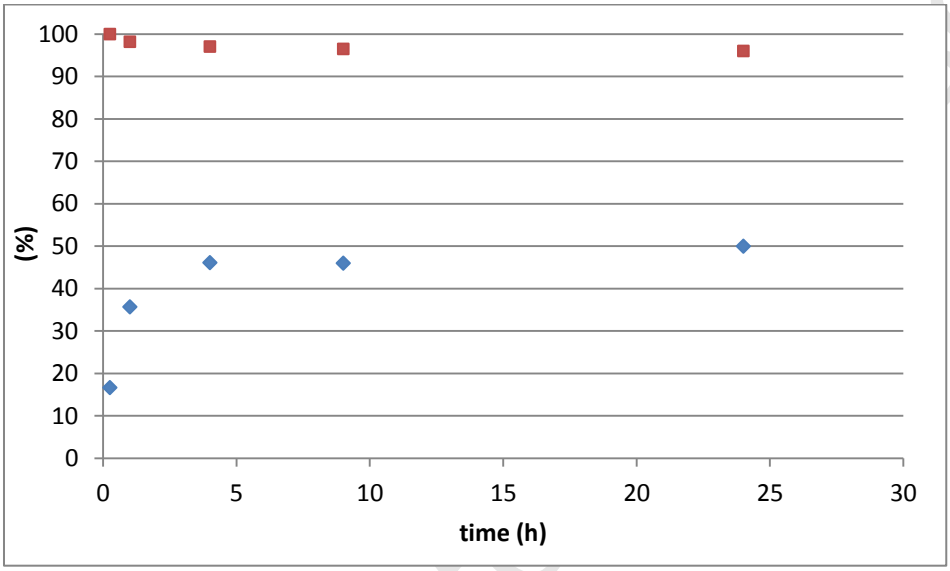


Fig. 5. Evolution of the conversion ◆ of 10 mM of 2-aminovaleronitrile (**2**) by NIT191 and enantiomeric excess ■ of (*S*)-norvaline formed after derivatization of the reaction medium with (*S*)-NIFE (see paragraph 2.5.1 for experimental conditions).

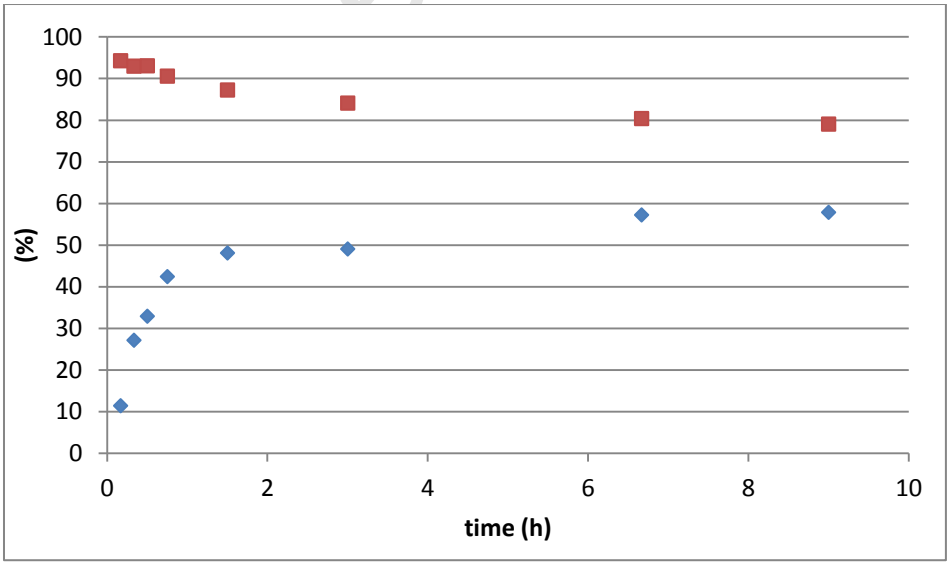


Fig. 6. Evolution of the conversion ◆of 10 mM of 2-amino-2-phenylacetonitrile (**5**) by NIT28 and enantiomeric excess ■ of (*R*)-phenylglycine formed (see paragraph 2.5.2 for experimental conditions).

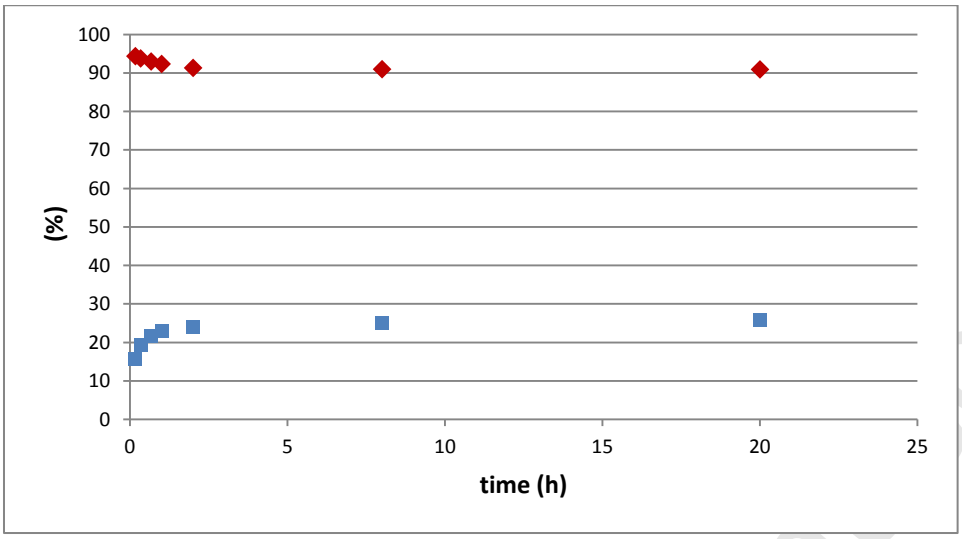


Fig. 7. Evolution of the conversion ◆ of 10 mM of 2-amino-2-(4-methoxyphenyl)acetonitrile (**9a**) by NIT28 and enantiomeric excess ■ of (*R*)-2-amino-2-(4-methoxyphenyl)acetic acid (**9b**) formed (see paragraph 2.5.4 for experimental conditions).

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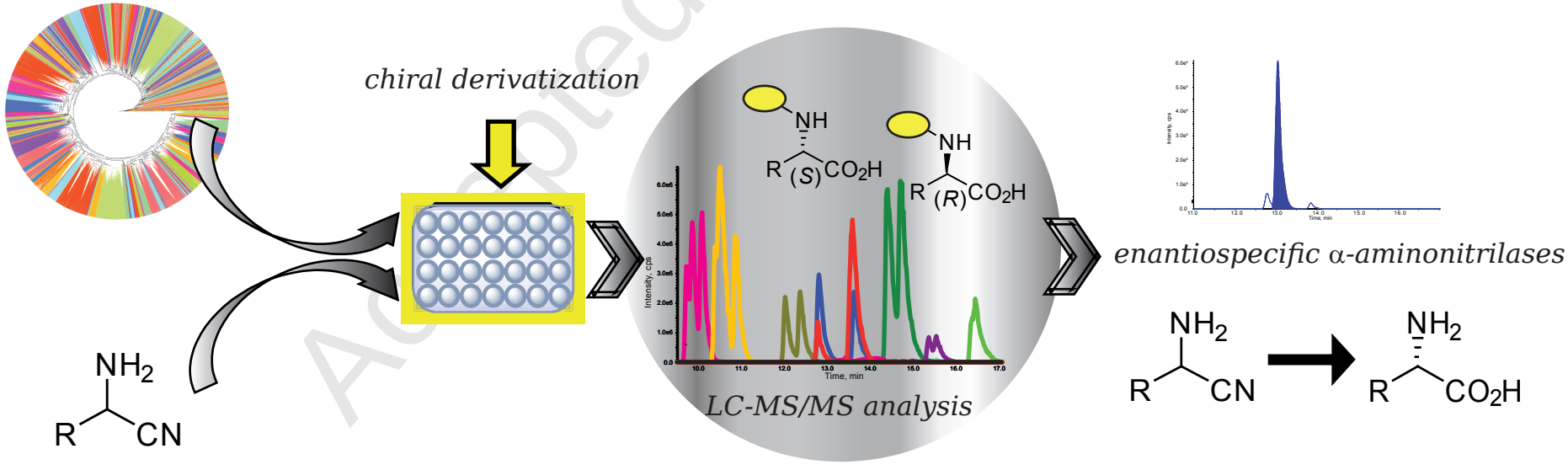

Highlights

We performed a large nitrilase activity screening on α-aminonitriles with diversified side chains (alkyl, polar, aromatic)

The large collection of tested enzymes covers the whole biodiversity of the nitrilase superfamily

An efficient liquid chromatography-electrospray ionization tandem mass spectrometry (ESI-MS/MS) method was carried out after derivatization of reaction mixtures to access both conversion rate and enantiospecificity of the nitrilase reaction

Five new α-aminonitrilases were found, with no nitrile hydratase activity observed.

(S)-norvaline, (R)-phenylglycine and two phenylglycine derivatives were obtained from corresponding α-aminonitriles