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Nitrilases Catalyze Key Step to Conformationally Constrained GABA Analogous γ-Amino Acids in High Optical Purity

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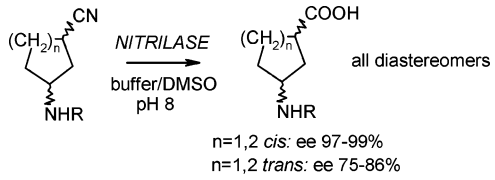

Five- and six-membered carbocyclic γ-amino acids were prepared in high enantiomeric purity by nitrilase-mediated transformation of hitherto unreported γ-amino nitriles. The nitrilases investigated reveal a strong enantiopreference for *cis*-isomers (up to 99% ee), whereas *trans*-isomers were available in up to 86% ee. The biocatalytic enantioselective syntheses of *cis*-3-aminocyclohexanecarboxylic acid (**3b**), *trans*-3-aminocyclohexanecarboxylic acids (**4b, 6b, 8b**) as well as *trans*-3-aminocylopentanecarboxylic acid (**2b**) are hereby reported for the first time.

γ-Amino butyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system (CNS).[1] As a highly flexible molecule it can participate in many low-energy conformation binding processes. In recent times, the application of conformationally restricted GABA mimics (mainly cyclic compounds containing a rigid backbone[2]) has contributed to a better understanding in GABA neuroreceptor research.[3] For example, 3-aminocyclopentanecarboxylic acid (3-ACPA) isomers were recognized to be particularly efficient stereomeric probes for GABA binding site topography. 3-Aminocyclohexanecarboxylic acid (3-ACHA), however, was found to act selectively as GABA uptake inhibitor.[4] In addition, analogues therefrom were investigated as therapeutic agents for a range of CNS disorders.[5,6] All these facts account for the need to elaborate synthetic procedures for enantiomerically pure cyclic GABA analogues.

Surprisingly, methods to prepare enantioenriched 3-ACPA and 3-ACHA can scarcely be found in the literature. The preparation of both enantiomers of *cis*-3-ACHA was achieved via classical fractional crystallization of the diastereomeric L-ornithine and brucine salts.[7] Both isomers of 3-oxo-CPA were obtained by resolution of their brucine adducts, following several transformation steps to either enantiomer of *cis*- and *trans*-3-ACPA.[8] The *cis* enantiomers were also prepared by resolution of their (−)-1-phenylethylammonium salts.[9] *cis*-(−)-3-ACPA was also isolated as a degradation product from the antiviral antibiotic amidinomycin.[10,11] In addition to these long-established methods, approaches to *cis*-3-ACPA comprise multiple-step chirospecific syntheses starting from aspartic acid,[12] asymmetric allylic alkylation of 4-azido-1-benzoyloxycyclopent-2-ene,[13] and the asymmetric reductive amination of (±)-3-oxocyclopentanecarboxylic esters.[14] In fact, few methods took advantage of the benefits of biocatalysis. Thus, only the enantiomers of *cis*-3-ACPA were obtained by esterase- and lipase-catalyzed desymmetrization of meso *cis*-1,3-cyclopentane dicarboxylic esters[11,15] and by lactamase-catalyzed kinetic resolution of a bicyclic lactam.[16] However, the enantioselective synthesis of *trans*-3-ACPA as well as either enantiomer of *cis*- and *trans*-3-ACHA has not yet been accomplished using the great resolution potential of biocatalysts.

For several years we have been interested in developing efficient chemoenzymatic methods for the synthesis of non-proteinogenic amino acids.[17,18] In this context, we present here our novel and practical access to enantioenriched γ-amino acids from racemic amino nitriles (Figure 1) by the use of nitrilase.

Nitrilases are hydrolases (EC 3.5.5.1) capable of enantioselectively hydrolyzing a nitrile group via several steps, albeit only the final product, the carboxylic acid, is released.[19] In the past, these enzymes have increasingly been applied for the enantioselective preparation of acids[20] because no intermediate product of hydrolysis (amide) is released from the catalytic site,[21] in contrast to the nitrile hydratase/amidase pathway. Incon-

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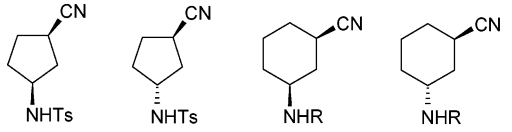

FIGURE 1. Novel (±)-γ-amino nitriles (a) for enzymatic transformations to enantioenriched γ-amino carboxylic acids (b); (only one enantiomer is depicted); Ts = toluenesulfonyl; Cbz = benzyloxycarbonyl; Bz = benzoyl

veniently, a serious restriction for the organic chemist when using these biocatalysts was the high efforts encountered so far, when carrying out the reactions in whole cell transformations. Hence, the recent availability of "ready for use" biocatalysts[22] simplifies the reaction protocol considerably.

To provide the hitherto unreported amino nitriles[23] (±)-**1a**−**8a**, we designed the straightforward procedures outlined in Scheme 1, including relevant modifications of reported approaches. Michael addition of cyanide to α,β-unsaturated cyclic ketones gave 3-oxo-cyclopentanecarbonitrile (±)-**9** and 3-oxo-cyclohexanecarbonitrile (±)-**10**. We made considerable improvements (see Supporting Information) compared with the poor yields achieved by the literature procedures for both five- and six-membered ketonitriles.[24−26] Subsequent $NaBH_4$ reduction of the ketones[27] afforded epimeric hydroxy nitriles in a *cis:trans*-isomer ratio of 88:12 for six-membered[24] (±)-**16** and (±)-**18** as well as 61:39 for five-membered rings (±)-**15** and (±)-**17**, determined by GC/MS analysis. Displacement of the mesylates of (±)-**15**−(±)-**18** by azide, reduction, and final protection gave the desired amino nitriles in Scheme 1. However, the minor formation of the *trans*-isomers (±)-**15** and (±)-**16** by the ketonitrile reduction renders this access unfavorable for *cis*-aminonitriles. We tested several conditions for reductive amination and found $NaCNBH_3$ to reduce the intermediate imines very efficiently to yield (±)-**11**−(±)-**14**.[28] The ratio of diastereomers, however, was close to equal. The diastereomers are separable on silica gel after application of the respective protecting groups. Alternatively, we found that *cis*-amino nitriles (±)-**1a** and (±)-**3a** can be obtained in high diastereoselectivity (30:1) by reductive amination using benzylamine as nitrogen donor and H_2/Pd/C in MeOH as the reduction system.

Initially, all γ-amino nitriles (±)-**1a**−**8a** (Figure 1) were subjected to nitrilase-mediated kinetic resolutions on a screening level to give acids **1b**−**8b**. To estimate the relative activity, the reactions were stopped after 18 h (for conversions and ee's see Table 1 in Supporting Information). The screening results suggest a preference of the nitrilases for the *cis*- versus the *trans*-isomers with respect to conversion as well as enantioselectivity. Evidently, the enzymes are sensitive toward changes in ring size; thus, five-membered carbonitriles react superiorly throughout as compared to six-membered substrates. *trans*-Carbonitriles were accepted exclusively by NIT-104 and NIT-107, except benzoate (±)-**8a**, which was not hydrolyzed by any of the nitrilases. The transformation of amino nitrile (±)-**5a** was accompanied by a loss of enantioselectivity compared to the tosylate (±)-**3a**. Replacement of the toluenesulfonyl group in *cis*-amino nitrile (±)-**3a** by benzoate (**7a**) decreases the conversions significantly (<18%). For example, NIT-106 gave the acid **3b** in 99% ee at 50% conversion, **5b** in only 38% ee at 8% conversion, whereas (±)-**7a** was not accepted as substrate at all. The reason for this obstacle of the benzoyl protection is unclear yet. With regard to different *N*-protecting groups, a clear trend for all the enzymes cannot be deduced. NIT-104 was more successful for **7b**, and NIT-105 for **5b** in high optical yield (99% ee and 94% ee, respectively), although both ee values were achieved at a point of low substrate conversion (Table 1 in Supporting Information). NIT-106 generally exhibited reverse enantioselectivity to all other applied nitrilases. Essentially, nitrilases NIT-101−NIT-103 and NIT-105 turned out to be unsuitable catalysts for the transformation of (±)-**1a**−(±)-**8a**.

On the basis of these screening results, optimized transformation protocols were established to synthesize particular acids on a preparative scale with the appropriate nitrilase in high optical purity (Table 1). The reactions were monitored by HPLC and stopped at the time of the maximal expected enantiopurity of the acids.[29] The yields given in Table 1 were determined after the isolation by extraction and chromatographic purification. The optical purities of the remaining nitriles and acids were determined by enantiomeric separation using HPLC; the resulting ee's are depicted in Table 1 (see also Experimental Section).

In agreement with the screening, nitrilases NIT-106 and NIT-107 are the most efficient catalysts with respect to the present compounds. Thus, *cis*-3-ACPA (+)-**1b** was prepared with the use of NIT-104, whereas (−)-**1b** was produced by NIT-106 in an enantiocomplementary manner in almost enantiopure form (97% ee) close to the theoretical yield of a kinetic resolution. The respective *trans*-isomer **2b** was obtained in only 55% ee by the same enzyme. All other nitrilases examined could not enhance this result. NIT-106 revealed similar outstanding selectivities in the transformation of six-membered aminonitrile *cis*-(±)-**3a** to (−)-**3b** in almost optical purity (>99% ee) and in 29% isolated yield at a comparable conversion with regard to that of *cis*-(±)-**1a** (Table 1). The enzyme's prerequisite for both high catalytic activity and enantioselectivity is best given by a 1,3-diequatorial conformation of the substituents, as present

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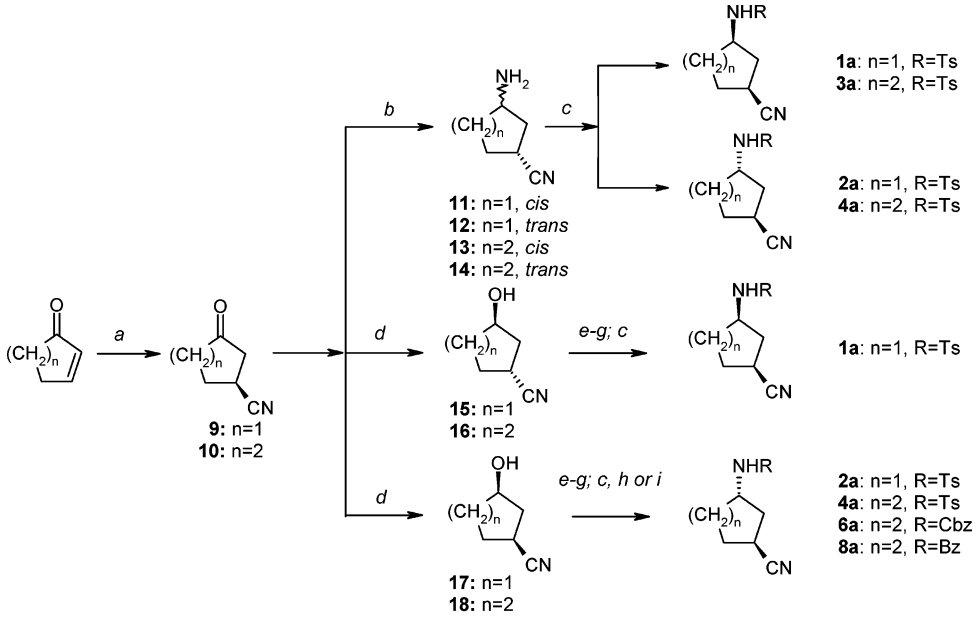
SCHEME 1. Synthesis of (±)-γ-Amino Nitriles (only one enantiomer is depicted)[a]



[a] Reagents and conditions: a) KCN, $Et_3N{\cdot}HCl$, $MeOH/H_2O$, 20 °C; b) I) ammonium formate, MS 4 Å, MeOH, rt; II) $NaCNBH_3$; c) TsCl, Et_3N, CH_3CN, rt;[30] d) $NaBH_4$, MeOH, rt; e) MsCl, Et_3N, CH_2Cl_2, 0 °C;[30] f) NaN_3, DMF, 60 °C; g) 1 bar H_2, Pd/C, MeOH, rt; h) BzCl, pyridine, CH_3CN, rt;[30] i) I) benzyl chloroformate, K_2CO_3, Et_2O/H_2O, 0 °C; II) rt.[30]

TABLE 1. Nitrilase Biohydrolysis of Racemic *N*-Protected γ-Amino Nitriles (a) to Enantioenriched γ-Amino Acids (b), Isolated Yields

substrate	enzyme	substrate (mM)	enzyme (g/L)	time (h)	conversion (%)	ee (%)	yield (%)
1a	NIT-106	0.56	0.50	1.75	47	97	45
2a	NIT-106	0.34	0.50	30	36	55	36
3a	NIT-106	0.42	0.50	9	42	>99	29
4a	NIT-107	0.67	1.00	256	46	86	46
6a	NIT-107	0.22	0.50	18.5	41	74	35

in *cis*-(±)-**1a** and *cis*-(±)-**3a**. Notably, the remaining nitriles were also recovered in up to 98% enantiomeric excess. Different from that, the respective *trans*-isomers **4b** and **6b** were obtained in good yields exclusively by NIT-107, although in diminished enantiomeric excess (86% and 74%, respectively). In this case, nitrilase NIT-106 completely failed to catalyze the transformations of *trans*-(±)-**4a** and *trans*-(±)-**6a**.[31]

The absolute configuration of *cis*-**1b** and *cis*-**3b** was assigned to be (1*R*,3*S*) by comparison of the HPLC-elution order with the only available reference acid *cis*-(1*R*,3*S*)-3-ACPA. By serendipity, this configuration matches that of the natural product, amidinomycin.[10,11] For a detailed description on the assignment of the other acids see the Supporting Information. With these results it became clear that NIT-106 acted highly *R*-selective in contrast to all other nitrilases applied in this study, which were *S*-selective throughout.

In summary, the nitrilase NIT-106-mediated hydrolysis of *cis*/*trans*-3-aminocyclopentane/hexane carbonitriles offers an efficient method for the enantioselective synthesis of (1*R*,3*S*)-*cis*-3-aminocyclopentanecarboxylic acid and its six-membered analogue (1*R*,3*S*)-*cis*-3-aminocyclohexanecarboxylic acid in high optical purity. In contrast, the best optical yield for the *trans*-isomer of 3-aminocyclohexanecarboxylic acid was achieved by NIT-107. The hereby presented approach represents the first enantioselective synthesis of *trans*-configured five- and six-membered ring carbocyclic γ-amino acids and their respective six-membered *cis*-diastereomers.

Experimental Section

The enzymes NIT-101−NIT-108 were purchased from a commercial supplier.[22] For preparative reactions, the enzymes used were delivered with the specifications: NIT-104 (3.7 U/mg solid), NIT-106 (85.0 U/mg solid), and NIT-107 (2.8 U/mg solid). For HPLC analysis a LiChrospher 100 RP-18e column (5 μm) was used. Chiral analysis was carried out with an Astec Chirobiotic R column, a Chromtech Chiral AGP 100.4 column (5 μm), a Daicel Chiralpak AD-H (5 μm), and a Chiralcel OD-H column (5 μm).

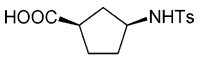
Screening. For screening experiments, 1.0 mg of enzyme preparation was dissolved in 497.5 μL of phosphate buffer (50 mM, pH 8.00, 1 mM EDTA), and 2.5 μL of the substrate was added as solution in MeOH or DMSO (40 mM) to give a final concentration of 0.2 mM. The reactions proceeded at 30 °C in a Thermomixer at 1100 rpm. After 18 h, 500 μL of acetone was added. The reaction vessels were centrifuged at room temperature and 13000 rpm for 5 min to remove precipitated proteins. The supernatant was analyzed by RP-18 HPLC using a gradient of 0.1% H_3PO_4 and acetonitrile. For ee determination on carbohydrate-based chiral stationary phases, the products were extracted using ethyl acetate. The solvent was removed and the residue dissolved in MeOH prior to chiral HPLC analysis.

Preparative Scale Biotransformations. The commercial enzyme preparation was dissolved in phosphate buffer (50 mM, pH 8.00, 1 mM EDTA[32]) in a round-bottomed flask. The substrate was added as solution in DMSO (max 5 vol % of cosolvent). The reaction

(30) For standard conditions, see: Greene, T. W.; Wuts, P. G. M. *Protective Groups in Organic Synthesis*, 3rd ed.; J. Wiley and Sons: New York, 1999.

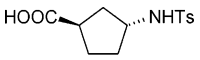
(31) Because no (published) crystal structure for any of the nitrilases is available now, such striking differences in substrate reactivity can not be rationalized. The same is true for the differing substrate specificity of NIT-107 towards amino nitriles **4a** and **6a**, resulting in remarkably different reaction times, depending on the protecting group

was stirred with a magnetic bar, and the temperature was adjusted to 29−31 °C by the use of an oil bath. The reaction was monitored by HPLC (conversion and enantiomeric excess, as described for the screening experiments). After completion, the products were isolated by extraction with ethyl acetate and purified by silica gel chromatography using ethyl acetate/cyclohexane mixtures with small portions of acetic acid. In cases where the protein content prevented the organic layer to clearly separate from the aqueous layer, the protein was removed by precipitation using $(NH_4)_2SO_4$ and filtration through a plug of Celite.



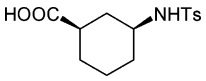
(1*R*,3*S*)-**1b**

***cis*-3-(Toluene-4-sulfonylamino)cyclopentanecarboxylic acid (1b):**[33] white solid, mp 127−129 °C; *NIT-104*: Yield 65 mg of (+)-(1*S*,3*R*)-**1b** (83% at 89% conversion, ee = 17%), $[\alpha]^{20}_D$ +0.6 (*c* 1.09, CH_2Cl_2) from 73 mg of (±)-**1a**; *NIT-106*: Yield 69 mg of (−)-(1*R*,3*S*)-**1b** (45% at 47% conversion, ee = 97%), $[\alpha]^{20}_D$ −24.6 (*c* 1.00, CH_2Cl_2) from 147 mg of (±)-**1a**; ^{1}H NMR ($CDCl_3$) δ 1.59−1.68 (m, 1H), 1.71 (tt, 1H, *J* = 5.1, 1.7 Hz), 1.74−1.82 (m, 1H), 1.85−1.97 (m, 2H), 1.98−2.04 (m, 1H), 2.43 (s, 3H), 2.82 (m, 1H), 3.76 (m, 1H), 5.54 (s, br, 1H, N*H*), 6.41 (s, br, 1H, COO*H*), 7.29 (d, 2H, *J* = 8.1 Hz), 7.75 (d, 2H, *J* = 8.1 Hz); ^{13}C NMR ($CDCl_3$) δ 21.8, 28.3, 33.7, 36.4, 41.6, 54.9, 127.3, 130.0, 138.0, 143.7, 181.7; *m*/*z* (EI) 201 (10), 173 (8), 155 (19), 91 (100), 67 (91); IR (CaF) 3238, 1705, 1598, 1322, 1158 cm^{-1}; Chiral separation on Chirobiotic R, polar organic mode (MeOH/Et_3N/AcOH, 100:0.1:0.4), 0.80 mL/min, 30 °C.



(1*R*,3*R*)-**2b**

***trans*-3-(Toluene-4-sulfonylamino)cyclopentanecarboxylic acid (2b):**[33] white solid, mp 139−141 °C; *NIT-106*: Yield 72 mg (81% at 90% conversion, ee = 6%) from 83 mg of (±)-**2a**; *NIT-106*: Yield 35 mg of **(+)-2b** (36% at 36% conversion, ee = 55%); $[\alpha]^{20}_D$ +0.2 (*c* 1.34, CH_2Cl_2) from 90 mg of (±)-**2a**; ^{1}H NMR ($CDCl_3$) δ 1.44−1.51 (m, 1H), 1.73−1.81 (m, 2H), 1.88−1.95 (m, 1H), 1.97−2.10 (m, 2H), 2.43 (s, 3H), 2.90 (m, 1H, H-1), 3.71 (m, 1H, *J* = 6.2 Hz, H-2), 5.07 (s, br, 1H, N*H*), 7.31 (d, 2H, *J* = 7.6 Hz), 7.75 (2H, d, *J* = 7.6 Hz), 8.45 (s, br, 1H, COO*H*); ^{13}C NMR ($CDCl_3$) δ 21.8, 27.7, 33.5, 36.6, 41.4, 54.8, 127.3, 130.0, 137.5, 143.8, 181.3; IR (CaF) 3270, 1705, 1599, 1321, 1157 cm^{-1}; Chiral separation on Chirobiotic R, polar organic mode (MeOH/Et_3N/AcOH, 100:0.1:0.4), 0.80 mL/min, 30 °C.



(1*R*,3*S*)-**3b**

***cis*-3-(Toluene-4-sulfonylamino)cyclohexanecarboxylic acid (3b):** white solid, mp 134−138 °C; *NIT-106*: Yield 55 mg of (−)-(1*R*,3*S*)-**3b** (48% at 50% conversion, ee = 88%), $[\alpha]^{20}_D$ −45.0 (*c* 1.09, CH_2Cl_2) from 113 mg (±)-**3a**; *NIT-106*: Yield 8.5 mg of (−)-(1*R*,3*S*)-**3b** (29% at 42% conversion, ee >99%), $[\alpha]^{20}_D$ −43.6 (*c* 0.355, CH_2Cl_2) from 23 mg of (±)-**3a**; ^{1}H NMR ($CDCl_3$) δ 1.07−1.14 (m, 1H), 1.22−1.29 (m, 2H), 1.39−1.42 (m, 1H), 1.78−1.84 (m, 2H), 1.91 (m, 1H), 2.10 (m, 1H), 2.30 (m, 1H), 2.43 (s, 3H), 3.10−3.13 (m, 1H), 4.76 (s, br, 1H, N*H*), 6.24 (s, br, 1H COO*H*), 7.30 (d, 2H, *J* = 8.1 Hz), 7.75 (d, 2H, *J* = 8.1 Hz); ^{13}C NMR ($CDCl_3$) δ 21.5, 23.9, 27.7, 33.4, 35.7, 41.9, 51.9, 126.9, 129.7, 138.1, 143.4, 179.4; *m*/*z* (EI) 215 (3), 186 (3), 173 (3), 155 (7), 124 (5), 91 (100); IR (CaF) 3271, 1705, 1599, 1321, 1157 cm^{-1}; Chiral separation on AGP, 10 mM phosphate buffer pH 7.01, 0.80 mL/min, 15 °C.

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Supporting Information Available: Details of the preparation of γ-amino nitriles, characterization data (**1a**−**8a**, **4b**−**6b**, **1c**−**6c**, and **8c**), enantiomer separation, determination of absolute configurations, screening results (**1a**−**8a**), results of additional preparative biotransformations (**1b**−**4b** and **6b)** as well as copies of all relevant NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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(32) Addition of DTT was omitted for reasons outlined in ref 21

(33) The racemic compound was mentioned in: Berger, H.; Paul, H.; Hilgetag, G. *Chem. Ber.* **1968**, *101*, 1525−1531. No spectral data are included.