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Realization of the synthesis of α,α-disubstituted carbamylacetates and cyanoacetates by either enzymatic or chemical functional group transformation, depending upon the substrate specificity of *Rhodococcus* amidase

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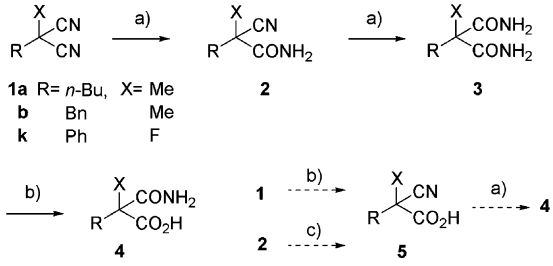
Abstract—Substrate specificity and enantioselectivity of nitrile hydratase and amidase from *R. rhodochrous* IFO 15564 has been studied by applying a series of α,α-disubstituted malononitriles and related substrates. The amidase preferentially hydrolyzed the *pro*-(*R*) carbamyl group (amide) of the prochiral diamides, an intermediate resulting from the action of nitrile hydratase in a non-enantiotopic group-selective manner. The introduction of a fluorine atom at the α-position caused an inhibitory effect on amidase. By a combination of this microbial transformation and the subsequent Hofmann rearrangement, an important precursor of (*S*)-methyldopa with 98.4% ee has been prepared. For the enzymatically poor substrate, the action on HO_3SONO–H_2O on the carbamyl group was effective, leaving the cyano group intact. This conversion is demonstrated as the key step for the expeditious preparation of (±)-α-cyano-α-fluoro-α-phenylacetic acid (CFPA) from diethyl α-fluoro-α-phenylmalonate.

Since the reaction of microbial nitrile hydratase and amidase on prochiral α,α-disubstituted malononitrile **1a** was disclosed in 1993, the substrate specificity has been studied on the structurally related cyanoacetamide and malonamide. In this reaction, the action of a multi-enzyme system consisting of nitrile hydratase and amidase on a bifunctional substrate (dinitrile) could provoke several possible intermediates and reaction pathways. Moreover, both of the enzymes might show different substrate specificity and enantioselectivity on each intermediate. Our initial studies revealed that the reaction pathway was somewhat complex (Scheme 1).[1] The activity of the nitrile hydratase involved in *Rhodococcus rhodochrous* IFO 15564,[2] on dinitrile **1a** itself as well as the corresponding primary product, cyanoamide **2a**, is very high, and, accordingly, the major intermediate, diamide **3a**, was obtained. The action of amidase on the prochiral diamide **3a** worked in an enantiotopic group-selective manner to give (*R*)-**4a** in high enantiomeric excess.



Scheme 1. Reagents and conditions: (a) Nitrile hydratase; (b) amidase; (c) nitrilase of *Rhodococcus*.

On the other hand, substrate specificity studies have been made by Wu and Li[3] using *Rhodococcus* sp. CGMCC 0497. Their results showed a similar tendency in the enantiotopic group-selective hydrolysis of amidase. In turn, the nitrile hydratase of this specific microorganism also worked in an enantiotopic

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Table 1. Hydrolysis of disubstituted malononitriles and related substances

Entry	Sub.	R	X	Time (d)	Product	Yield (%)	Ee[7] (%)	Config.[7]
1	**1c**	Et	Me	0.2	**4c**	85	12	*S*
2	**1d**	Pr	Me	1	**4d**	81	75	*R*
3	**1e**	Allyl	Me	1	**4e**	72	95	*R*
4	**1a**	Bu	Me	1	**4a**	92	96	*R*
5	**1f**	CH_2=$CH(CH_2)_2$–	Me	1	**4f**	76	49	*R*
6	**1g**	*i*-Pr	Me	4	**4g**	84	23	—
7	**1h**	Ph	Me	7	**4h**	48	>99	*R*
8	**1b**	$PhCH_2$–	Me	3	**4b**	90	>99	*R*
9	**1i**	3,4-$(OCH_2O)C_6H_3CH_2$–	Me	3	**4i**	95	98	*R*
10	**1j**	Bu	Bu	1	**3j**	74	—	—
11	**1k**	Ph	F	1	**3k**	26	—	—
					4k	16	13	ND
					5k	40	10	*R*
12	**1k**	Ph	F	21	**3k**	58	—	—
					4k	4	56	ND
13	**3k**	Ph	F	5	**3k** (recov.)	52	—	—
					4k	4	31	ND
14	**2k**	Ph	F	3[8]	**2k** (recov.)	Quant.	—	—

group-selective manner on dinitrile **1b** to give (*S*)-**2b** under certain conditions.

This situation prompted further interest in the selective reactions of the enzymes; however, the present conclusion has been made based on only a few substrates while the detailed specificity still remains unknown. While Wu and Li's studies were limited to aromatic compounds, in our work, no substrates with different substituents other than **1a** were investigated. Needless to say, a concise estimation of the scope and limitations of enzyme-catalyzed reactions plays a crucial role in deciding whether enzymatic functional transformation or functional transformation is applied, to realize the goal toward the α,α-disubstituted carbamylacetic and cyanoacetatic acids of important use and application. Herein we report the substrate specificity of *R. rhodochrous* IFO 15564[4–6] with a series of substrates possessing a wide range of substituents.

Table 1 summarizes the result on the substrate specificity with dinitriles **1a**–**k**. Unless otherwise stated, the reaction was worked up when the substrate was consumed. In most cases, carbamylacetic acids **4** were the major products. The rate of the hydrolysis was greatly affected by the steric bulkiness around the susceptible functional groups. Compared with linear aliphatic substrates (entries 1–5), it took longer reaction times for the completion of the hydrolysis of aromatic substrates (entries 7–9). The substrate specificity of the nitrile hydratase was found to be rather broad, as all the dinitriles were quickly converted to the corresponding diamides. In the case of sterically hindered substrates **1g** and **1h** (entries 6 and 7, respectively), the corresponding diamides **3g** and **3h** could be clearly detected during the incubation. Except for one example where there was a small difference in bulkiness between the methyl and ethyl groups (**1c**, entry 1), the *pro*-(*R*)-carbamyl group on **3** was preferentially hydrolyzed by the amidase. The highly sterically hindered compound **1j** (entry 10) with two butyl groups on the malononitrile was a very poor substrate for amidase to give mainly the diamide (74%). The results suggest that the amidase may require at least one small α-substituent (ethyl group being mostly preferred) for its substrate to be hydrolyzed. The enantiotopic group selectivity of the amidase is quite understandable, when taking into account the configuration adjacent to the carbamyl group into account.

It is also supposed that there exists a certain interaction between the amidase and the π-electron system of the side chain on the substrates, as the changes in ee of products **4** depend upon both the chain length and the presence or absence of a double bond in the side chain (**1d** and **1e**, entries 2 and 3; **1a** and **1f**, entries 4 and 5). This tendency is consistent with the fact that high ees were shown by the products with aromatic side chains (**1b**,**h**,**i**, entries 7–9).

Substitution of the methyl group in **1h** by a strong electron-withdrawing fluorine atom brought about a rather confusing result. The reaction of **1k** (entry 11) yielded a complex mixture; diamide **3k** (26%) and the desired product **4k** (16% yield, 13% ee), along with a cyanoacetate **5k** in 40% yield with low ee [(*R*); 10%]. This conversion, however, was not reproducible. In another run (entry 12), the reaction was very slow with the products being **3k** (58%) and **4k** (4%, 56% ee). An independent experiment (entry 13) indicated that diamide **3k** (entry 13) was indeed a poor substrate for amidase. A plausible pathway to yield cyanoacetate **5k** from **1k** (entry 11) was supposed to be the sequential action of nitrile hydratase and amidase via an intermediate, cyanoamide **2k** (Scheme 1). We then submitted it to the microbial hydrolysis (entry 14),[8] but to our disappointment, no hydrolysis of the carbamyl group on **2k** was observed, either. Moreover, monoamide **2k** and diamide **3k** themselves worked as inhibitors for the amidase.[9] A characteristic feature commonly observed with **2k** and **3k** was the imino-enol form **2k′** and **3k′** (Fig. 1) to some extent.[10] Sharp absorbance at 3415 (**2k′**) and 3460 (**3k′**) cm^{-1} was not observed in the nonfluorinated derivative **3l**. Compounds **2k′** and **3k′** may play important roles through some unusual interaction

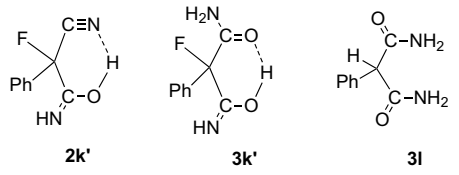
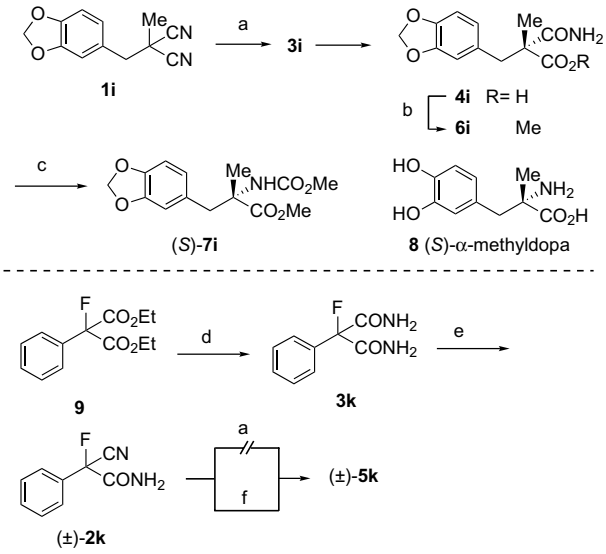


Figure 1.

between the compounds and the amidase at its active site, although the enol forms are limited in crystalline states and all three compounds exist as the conventional amide forms under NMR measurement conditions in DMSO. The reason for the formation of cyano acid **5k** under microbial treatment could be the participation of a nitrilase, which has so far not been observed in this microorganism.[11]

Based on this substrate specificity study, we then embarked upon the preparation of an important precursor, (*S*)-**7i**,[12] of (*S*)-α-methyldopa **8** as shown in Scheme 2. Malononitrile **1i** was incubated with the cultured cells of *R. rhodochrous* while subsequent methylation of carboxylic acid **4i** gave (*R*)-**6i**[13] with 98.2% ee in 95% yield. A Hofmann rearrangement of the further recrystallized sample (99.8% ee) followed by trapping with methanol provided (*S*)-**7i**[14] with 98.4% ee in 73% yield.

Another target was CFPA **5k**,[15] a useful chiral NMR derivatizing reagent. As both enantiomers are required due to the application of CFPA and enantiomeric resolution of itself and closely related compounds has so far been established, a new and expeditious route to its racemate is of the considerable importance. Due to the substrate specificity as above, we did not persist in the enzymatic hydrolysis, but concentrated instead on



Scheme 2. Reagents and conditions: (a) *R. rhodochrous*; (b) CH_2N_2; (c) Br_2, MeONa/MeOH; (d) liq NH_3; (e) Burgess' reagent; (f) HSO_3ONO–H_2O.

the chemical conversion of the carbamyl to a carboxyl group (from **2k** to **5k**, Scheme 2). Through a series of studies on the preparation of the substrates as above, we established the preparation of (±)-**2k** as follows. A known diester **9**[16] was treated with liquid ammonia to give the diamide **3k**[17] (81%). Subsequently, the dehydration of one of the two carbamyl groups efficiently proceeded to give (±)-**2k** (80%).[18] Finally, hydrolysis via activation of the carbamyl group under the treatment with nitrosylsulfuric acid was quite effective to give (±)-CFPA **5k**[19] (72%) leaving the cyano group in **2k** intact.

In summary, this detailed study on the substrate specificity of the nitrile hydratase and amidase of *R. rhodochrous* IFO 15564 and the inhibitory effect for the latter, was informative to provide (*S*)-**7i** (98.4% ee) by enzymatic functional group transformation as well as desymmetrization and an expeditious chemical synthesis of (±)-CFPA **5k** from **9** (three steps, 47%).

Acknowledgements

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2. Formerly *R. butanica* ATCC 21197.[1] *R. rhodochrous* IFO15564 is now available as the strain of NBRC15564 from the NITE Biological Resource Center (NBRC), 2-5-8, Kazusakamatari, Kisarazu, Chiba 292-0818, Japan.
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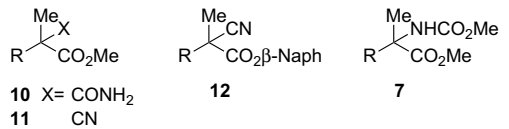
7. The ees were estimated either by the HPLC analyses of **10**, **11**, or **12**. The absolute configuration of the products was determined by the derivation to known **7**, the corresponding α-amino acids and/or mutual conversion between the products



8. The recently developed conditions were applied, using DMF as co-solvent (3.8% v/v) at an elevated temperature (45 °C), under which the nitrile hydratase was selectively deactivated,[6] to avoid the formation of the enzymically inactive **3k**.
9. The amidase activity in *Rhodococcus* was assayed in a reaction mixture with a total volume of 1.62 mL containing 2-naphthylacetamide (2 mg, 10.8 μmol), dissolved in DMF (60 μL), 0.1 M KH_2PO_4–Na_2HPO_4 buffer (pH 6.0) and *R. rhodochrous* (wet cells, 46 mg) and then incubated at 30 °C for 10–60 min with or without **2k** (2 mg, 11.2 μmol). The progress of the reaction was monitored by HPLC analysis of the appropriately pre-treated crude mixture [ODS, 25 cm × 4.6 mm; solvent, 10 mM H_3PO_4–KH_2PO_4 buffer solution (pH 2.8)/acetonitrile = 3/2; flow rate, 1.0 mL/min; detection at 254 nm]. 7.5 min (2-naphthylamide), 8.3 min (**2k**), and 15.4 min (2-naphthylacetic acid). The conversion of 1-naphthylamide to 2-naphthylacetic acid was 17% at 30 min in the presence of **2k**, while 60% conversion was recorded in the control experiment. The relative activity under the influence of **2k** was 28%. In a similar manner, the activity decreased to 70% by **3k**.
10. IR (KBr) **2k**: 3415, 3311, 3184; **3k**: 3460, 3344, 3206; **3l**: 3357, 3195 cm^{-1}. ^1H NMR (DMSO-d_6) δ **2k**: 8.38 (s, 1H), 8.57 (s, 1H); **3k**: 7.80 (s, 4H); **3l**: 7.64 (s, 4H). No noticeable changes were observed by the addition of D_2O except for the quick exchange of amide protons.
11. Another result supporting the contribution of Fe(III) independent nitrilase is as follows: The introduction of EDTA (0.1 M) or EDTA–Na (0.3 M) retarded the whole-cell nitrile hydratase-mediated formation of amide in cyclohexene-4,5-bis-acetonitrile, another good substrate, the cyanoacid however, increased. Cf. Matoishi, K.; Sano, A.; Imai, N.; Yamazaki, T.; Yokoyama, M.; Sugai, T.; Ohta, H. *Tetrahedron: Asymmetry* **1998**, *9*, 1097–1102.
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13. Pre-cultivation of the microorganism and the incubation of the substrate **1i** was according to the reported procedure.[5,6] and the enzyme activities were confirmed as above.[9] Mp 112.0–112.5 °C; $[\alpha]_D^{19} = +4.9$ (*c* 1.01, $CHCl_3$), $[\alpha]_D^{22} = -7.3$ (*c* 0.75, EtOH) {lit.[12] $[\alpha]_D^{25} = -4.6$ (*c* 0.63, EtOH)}; ^1H NMR δ 1.42 (s, 3H), 3.02 (d, 1H, $J = 13.4$ Hz), 3.27 (d, 1H, $J = 13.4$ Hz), 3.74 (s, 3H), 5.91 (s, 2H), 5.93 (br, 1H), 6.58 (d, 1H, $J = 8.1$ Hz), 6.63 (s, 1H), 6.69 (d, 1H, $J = 8.1$ Hz), 6.89 (br, 1H); ^{13}C NMR δ 20.8, 43.3, 52.6, 55.2, 100.9, 108.0, 110.2, 123.1, 130.1, 146.6, 147.5, 173.5, 174.6; IR (KBr) 3420, 3190, 1730, 1685, 1625, 1485, 1440, 1355, 1245, 1200, 1120, 1035, 930, 865, 825, 770, 715, 660, 590 cm^{-1}. Anal. Calcd for $C_{13}H_{15}NO_5$: C, 58.86; H, 5.70; N, 5.28. Found: C, 58.78; H, 5.78; N, 5.37. Its ee was determined by the HPLC analysis; column, CHIRALCEL OJ; hexane/2-propanol = 9/1; flow rate, 0.5 mL/min; t_R (min) 40.9 (0.9%, for *S*), 47.9 (99.1% for *R*).
14. Bp 185–190 °C/ 3 torr (bulb-to-bulb distillation); $[\alpha]_D^{22} = +55.3$ (*c* 1.08, $CHCl_3$); $[\alpha]_D^{24} = -27.9$ (*c* 0.57, EtOH) {lit.[12] $[\alpha]_D^{25} = -24$ (*c* 0.598, EtOH)}; ^1H NMR δ 1.61 (s, 3H), 3.08 (d, 1H, $J = 13.8$ Hz), 3.32 (d, 1H, $J = 13.8$ Hz), 3.68 (s, 3H), 3.76 (s, 3H), 5.44 (br s, 1H), 5.92 (s, 2H), 6.50 (d, 1H, $J = 1.7$, 7.8 Hz), 6.52 (d, 1H, $J = 1.5$ Hz), 6.71 (d, 1H, $J = 7.8$ Hz); ^{13}C NMR δ 23.6, 41.7, 52.0, 52.7, 60.8, 100.9, 108.1, 110.1, 122.9, 129.7, 126.6, 147.6, 155.4, 174.1. Anal. Calcd for $C_{14}H_{17}NO_6$: C, 56.94; H, 5.80; N, 4.74. Found: C, 56.66; H, 5.83; N, 4.52. HPLC analysis; column, CHIRALCEL OJ; hexane/2-propanol = 19/1; flow rate, 0.5 mL/min; t_R (min) 50.4 (99.2%, for *S*) and 55.1 (0.8% for *R*).
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17. Compound **3k**: Colorless needles, mp 224–225 °C.
18. To a solution of diamide **3k** (1.01 g, 5.15 mmol) dissolved in anhydrous CH_3CN (80 mL), methyl *N*-(triethylammonium-sulfonyl)carbamate (Burgess' reagent, 760 mg, 3.19 mmol) was added. At intervals of 30 min under stirring at 60 °C, portions of the reagent (517 mg, 2.17 mmol, and 490 mg, 2.05 mmol) were added, respectively. Conventional work-up and purification gave **2k** (723 mg, 80%) as colorless needles, mp 111.5–112 °C; ^{13}C NMR (DMSO-d_6) δ 89.3 (d, $J = 200$), 115.2 (d, $J = 33$), 125.2 (d, $J = 5$, ×2), 129.2 (×2), 131.0, 132.0 (d, $J = 22$), 164.1 (d, $J = 24$). MS (*m*/*z*) 179 (M^++1, 5%), 159 (M^+-H_2O, 6%), 135 (100%), 108 (97%), 77 (5%), 44 (64%).
19. In a similar manner to the reported procedure,[5,20] to a solution of **2k** (300 mg, 1.68 mmol) in DMF (3 mL) was added HO_3SONO (645 mg, 5.07 mmol) and the mixture was stirred for 1 h at room temp. Conventional work-up and purification afforded **5k** (216 mg, 72%). ^1H NMR ($CDCl_3$) δ 7.49–7.66 (m, 5H), 10.16 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 86.8 (d, $J = 199$), 113.4 (d, $J = 33$), 125.4 (d, $J = 5$, ×2), 129.3 (×2), 131.4, 164.1 (d, $J = 30$); IR (film) 3505, 2254, 1768, 1600 cm^{-1}. The ^1H NMR and IR data were in good accordance with those reported previously.[15b]
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