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Tetrahedron 60 (2004) 607–618
Tetrahedron

Preparation of N-unsubstituted β-ketoamides by *Rhodococcus rhodochrous*-catalysed hydration of β-ketonitriles

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Received 20 June 2003; revised 25 July 2003; accepted 17 October 2003

Abstract—A varied set of N-unsubstituted β-ketoamides, hardly obtainable or non-accessible by non-enzymatic methods, have been synthesized, with good to excellent yields, by the generally fast hydration of the corresponding β-ketonitriles, catalysed by the bacterium *Rhodococcus rhodochrous* IFO 15564. This bacterium shows nitrile hydratase and amidase activities, the latter being inhibited during its growth phase with diethyl phosphoramidate (DEPA). Optimization of the processes and studies concerning large-scale biotransformations were also carried out. β-Ketoamides exist as keto–enol mixtures whose composition depends on their substituents and varies with solvent polarity.

1. Introduction

β-Ketoamides are versatile intermediates for the synthesis of heterocycles, for instance 3-acyltetramic acids[1] (used in the total synthesis of tirandamycin[2] and other related natural antibiotics), pyrans,[3] alkaloids,[4] lactams and spirolactams,[5] and azetidin-2-ones,[6] as well as several 3-hydroxyisothiazol bioisosteres of glutamic acid and analogs of the AMPA receptor agonist.[7] Recently, some β-ketoamides have been converted into γ-ketoamides, a class of compound related with a wide variety of biologically relevant systems.[8]

In connection with our current interest in the bioreduction of β-ketoamides and their subsequent transformation into non-racemic β- and γ-aminoalcohols,[9] we needed a set of N-unsubstituted β-ketoamides as starting materials. This type of β-ketoamide is hardly accessible by means of their usual preparative methods,[10] mostly directed towards the N-substituted ones. For such a cause, we decided to prepare them via the hydration of the corresponding β-ketonitriles, a commercially or easily available kind of compound. After a series of failed attempts using traditional, non-enzymatic methodologies, we envisaged that enzymatic hydration of β-ketonitriles would be a suitable, environmentally benign way of reaching our objective.

The enzymes involved in the desired biotransformation, i.e. nitrile hydratases (EC 4.2.1.84), are not commercially available, which forced us to use an appropriate whole cell culture. However, the bacterial strains able to hydrolyze nitriles can act according to one or both of the following routes: (i) a nitrile hydratase converts the nitrile into an amide, which is subsequently transformed into a carboxylic acid by an amidase; (ii) a nitrilase directly converts the nitrile into an acid. The presence of amidases and/or nitrilases is a serious drawback for our purposes of stopping the process in the amide stage, and it should be avoided.

A solution would be the use of some bacterial strains (*Rhodococcus* sp. N774, *Pseudomonas chlororaphis* B23, *Rhodococcus rhodochrous* J1) which develop nitrile hydratases almost exclusively when growing under appropriate conditions. Consequently, they have been used for an important industrial biotransformation (Nitto Chemical Industry Co.) leading to acrylamide, as well as for the preparation of other commodity chemicals.[11] One of these strains (*P. chlororaphis* B23) has also been employed for the industrial conversion of adiponitrile to 5-cyanovaleramide (DuPont),[12] as the first step in the manufacture of a new herbicide. Unfortunately, these strains are commercially unavailable.

Apart from these examples, the scarcely reported bacterial transformations of nitriles into amides[13] seem to be related to an accidental, unfavourable steric interaction between the amidase and the corresponding amide, which slows down its further conversion to acid. Thus, several *ortho*-substituted benzonitriles (but not their *meta*- or *para*-isomers) and five membered hetarene-2-carbonitriles were transformed into

Keywords: Amido-ketones; Enzyme inhibitors; Microbial reactions; *Rhodococcus rhodochrous*; Tautomerism.
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0040–4020/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2003.10.096

their corresponding amides after short reaction times with *R. rhodochrous* AJ270,[14] another inaccessible strain. In the same way, the hydration carried out with *R. rhodochrous* IFO 15564 of three cyanocarbohydrate derivatives to amides,[15] as well as that of 3-cyanoprop-1-enylboronic acid,[16] can probably be rationalised.

On the other hand, it has recently been demonstrated that diethyl phosphoramidate [$H_2N-P(O)(OEt)_2$; DEPA] behaves as an amidase inhibitor for three nitrile hydrolyzing bacterial strains when exposed to 2-arylpropanenitriles and to 2-arylpropanamides. With *Agrobacterium tumefaciens* strain d3, 2-arylpropanenitriles were enantioselectively converted into 2-arylpropanamides,[17] as the hydration of the nitrile to amide was not inhibited by DEPA;[17b] similar results were observed for the strains *Rhodococcus equi* A4[18a] and *Rhodococcus* sp. MP50.[18b]

The above observations prompted us to undertake the biotransformations of a varied set of β-ketonitriles into their corresponding β-ketoamides. This paper describes our findings with the concourse of the bacterium *R. rhodochrous* IFO 15564 in metabolic resting phase. Since the obtained β-ketoamides are also accepted as substrates by this strain, we have reached our goal by using DEPA as amidase inhibitor. To the best of our knowledge, bacterial hydration of β-ketonitriles has not been previously performed for preparative purposes.

2. Results and discussion

2.1. Choice of the starting β-ketonitriles

Eighteen varied β-ketonitriles, R^1-CO–CHR^2–CN (**1**), were selected for this study (see Table 3). Sixteen of them (**1a**–**r**) show no substituent at α position (i.e., R^2=H). Three of them have R^1 of aliphatic nature (**1a**–**c**), although **1c** includes a hetarene ring in R^1. The following eight (**1d**–**k**) possess aromatic R^1 groups, with or without electron-donor or electron-withdrawing substituents. In five cases (**1m**–**r**), R^1 is heterocyclic, both π-excessive and π-deficient, as well as mono- or bicyclic. Last, two starting materials (**1s**,**t**) are α-substituted, showing their R^1 and R^2 groups having interchanged aliphatic and aromatic nature. Some of them (**1c**,**m**,**n** and, in lesser extent, **1r**) are acid-labile. Such a selection seems to be wide enough to test the versatility of the biotransformation, as well as to compare with the following non-enzymatic reactions.

2.2. Non-enzymatic attempts

A variety of conventional reactions to transform nitriles into their corresponding amides were tried in order to find a general method for a wide range of β-ketonitriles **1** (see Table 1). First, the classical hydration using hydrogen chloride dissolved in concentrated hydrochloric acid was employed,[19] which led to acceptable results with the acid-stable starting material 3-oxo-3-phenylpropanenitrile (benzoylacetonitrile, **1d**; entry 1), but not with acid-labile heterocyclic substrates (entries 2 and 3). Another recent, mild and acid mediated way of converting nitriles into amides implies the use of chlorotrimethylsilane–water.[20] When applied on β-ketonitriles, it worked acceptably on **1d** again (entry 4), but not on the presumably acid-stable pivaloylacetonitrile **1b** (entry 5), and even less on the acid-labile heterocyclic **1m** (entry 6).

It can be predicted that hydration of β-ketonitriles under basic conditions would not work, since the abstraction of the acidic α-hydrogen will prevent a further nucleophilic attack to the cyano group.[21] Indeed, using **1d** as a test substrate, we observed no reaction after heating with potassium hydroxide in *tert*-butyl alcohol (entry 7),[22] and neither with potassium trimethylsilanolate in tetrahydrofuran (entry 8).[23] Last, the use of basic hydrogen peroxide under phase-transfer catalysed conditions (entry 9)[21] converted **1d** into benzoate anion, presumably via a retro-Claisen type reaction, opposite to that used by us for preparing some of the β-ketonitriles employed in this study (see Section 3.2).

Two enzymatic reactions with an acid-stable (entry 10) and an acid-labile β-ketonitrile (entry 11) were also included in Table 1 for comparison. From their results, it can be advanced that the bacterial biotransformation is the method of choice for hydrating β-ketonitriles to β-ketoamides, as will be plain in the sections to come.

2.3. Bacterial strain selection

After checking that no conventional procedure was able to convert the majority of β-ketonitriles into β-ketoamides, we decided to submit them to microbial hydration. As previously stated, except for a few anecdotal cases,[24] the overwhelming majority of known nitrile-converting microorganisms are bacteria. After discarding those exhibiting nitrilase activity, our attention was focused on four of them available at some culture collection, and able to transform nitriles via nitrile hydratase and amidase activities: *Brevibacterium imperiale* B222 (CBS 498.78),[13f] *Rhodococcus* sp. R312 (CBS 717.73),[25,26] *Rhodococcus erythropolis* IFO 12539[27] and *R. rhodochrous* IFO 15564.[15,16,28]

Benzoylacetonitrile (**1d**) was chosen as a reference substrate to evaluate the enzymatic activities of these four strains. Bacteria were grown both in the presence and in the absence of DEPA. Incubations with **1d** were carried out with resting phase bacteria and monitored by TLC. With *B. imperiale* neither benzoylacetamide (**2d**) nor benzoylacetic acid (**3d**) were detected (see Scheme 1). Amidase from *Rhodococcus* sp. R312, after growing this bacterium in the absence of DEPA, was much more active than the corresponding nitrile hydratase as only **1d** and **3d**, but not the ketoamide **2d**, were observed during the biotransformation. The amidase activity of the bacterium clearly decreased if growing in the presence of DEPA, although not enough to prevent a considerable fall in the ketoamide yield.

On the contrary, *R. rhodochrous* has been described to show higher nitrile hydratase than amidase activity. In fact, in our hands both this strain and *R. erythropolis* gave the best results in transforming **1d** into **2d**, especially after growing them in the presence of DEPA. Under certain reaction conditions, they allow the isolation of high yields of the ketoamide **2d**, the ketonitrile **1d** and the ketoacid **3d** being observed only in very small or null amounts. A comparative

Table 1. Non-enzymatic attempts at hydrating[a] R^1-CO–CHR^2–CN (**1**)[b] to R^1-CO–CHR^2–$CONH_2$ (**2**)

Entry	Substrate[c]	R^1	Reagents	Reaction time (h)	**2**, Crude yield (%)/remarks
1	**1d**	Ph	HCl(g)/HCl(aq)	13	70
2	**1m**	2-Furyl	HCl(g)/HCl(aq)	13	13/Notable polymerisation
3	**1c**	(1-Methylpy-rrol-2-yl)methyl	HCl(g)/HCl(aq)	13	0/Full polymerisation
4	**1d**	Ph	Me_3SiCl/H_2O	23	71
5	**1b**	Bu^t	Me_3SiCl/H_2O	39	36
6	**1m**	2-Furyl	Me_3SiCl/H_2O	39	5
7[c]	**1d**	Ph	KOH/Bu^tOH	2	No reaction
8[d]	**1d**	Ph	$Me_3SiO^-K^+$/THF	22	No reaction
9	**1d**	Ph	H_2O_2/NaOH(aq)/$Bu_4N^+Cl^-$	2	Retro-condensation to $PhCO_2H$
10[e]	**1d**	Ph	*R. rhodochrous*/H_2O	0.67	96
11[e]	**1m**	2-Furyl	*R. rhodochrous*/H_2O	1.2	90

[a] The reactions were carried out at room temperature except for entries 7, 8, 10 and 11.
[b] The four substrates selected for this table have R^2=H.
[c] Reaction temperature, 83 °C.
[d] Reaction temperature, 66 °C.
[e] Enzymatic process included for the sake of comparison; reaction temperature, 28 °C.

analysis of the ^{1}H NMR spectra of the crude products allowed us to select *R. rhodochrous* IFO 15564 as the most suitable bacterial strain for the enzymatic hydration of β-ketonitriles to β-ketoamides.

2.4. Optimization of the growth and biotransformation conditions

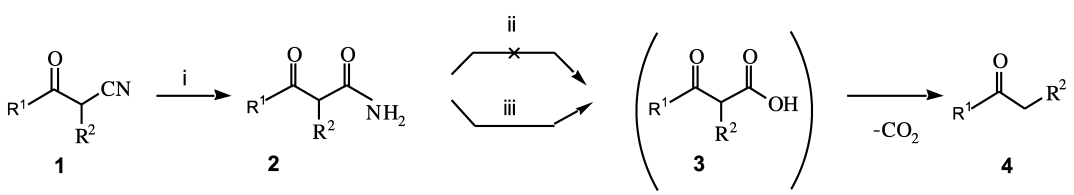
In order to determine the optimal conditions for obtaining the best yield of benzoylacetamide (**2d**) from benzoylacetonitrile (**1d**), as well as to check the amidase inhibitory power of DEPA, we carried out a series of biotransformations (see Table 2). Half of these processes were carried out with *R. rhodochrous* grown in the presence of DEPA, and the other half in the absence of this amidase inhibitor. The percentage values of compounds **1d** and **2d** were deduced from the ^{1}H NMR spectra of the crude reaction products, which were obtained from the cell-free supernatant reaction medium by means of continuous extraction and further solvent elimination. This work-up precludes the possibility of isolating benzoylacetic acid, **3d** (or other β-ketoacids **3** when other substrates were used), since the extractions were carried out at pH 8. No efforts were made for detecting β-ketoacids **3**; however, their undesirable formation was proved: (1) by TLC monitoring of the bioconversions;[29] (2) by the presence in some crude products of the ketones **4**, coming from the decarboxylation of the corresponding **3** during the biotransformation at 28 °C[30] (see Scheme 1).

From the above paragraph, it is clear that, in each process contained in Table 2, the difference $100-\Sigma'$ roughly represents the molar percentage of β-ketoacid coming from the amidase activity of the bacterium. Thus, by comparing both columns of Σ' values, the efficiency of DEPA as amidase inhibitor can be estimated from the $\Delta\Sigma'$ values, ranging from 14% (first row) to 27% (sixth row) of avoided β-ketoacid formation. However, DEPA also shows an unexpected feature as promoter of the nitrile hydratase activity, as deduced from the comparison of both **1d**-columns: the average reaction rate (rows 1 to 4) of the ketonitrile to ketoamide step is ca. 18% higher when the bacterium was grown in the presence of DEPA. Thus, the actual amidase inhibitory power of DEPA is higher than that merely inferred from the $\Delta\Sigma'$ values since, with its mediation, less β-ketoacid is formed just when more β-ketoamide is present in the reaction medium.

Apart from the obvious need of growing *R. rhodochrous* in the presence of DEPA, the ideal reaction conditions for preparing benzoylacetamide (**2d**) must include an adequate balance between the nitrile hydratase concentration (i.e., the absorbance or optical density of the bacterial suspension) and the reaction time. Data of Table 2 show that an A_{650} (absorbance of the bacterial suspension at λ=650 nm) value of 3.0 and a reaction time of 40 min lead to an excellent yield of **2d**. Data of row 6, if compared with those of row 3, clearly prove how longer reaction times favours the formation of β-ketoacid **3d**, which is an evident disadvantage in order to reach a good ketoamide yield.

Among the standard reaction conditions for the other substrates, we adopted the above A_{650} value of 3.0, the biotransformations being stopped after TLC showed the disappearance of the corresponding β-ketonitrile **1**. The use of more concentrated bacterial suspensions causes some problems of entrapping reaction products. Nevertheless, this method or other variations were applied when necessary, as described in the next Sections 2.5 and 2.6.

It has been described that DEPA acts as an amidase inhibitor



Scheme 1. (i) *R. rhodochrous* IFO 15564 (nitrile hydratase activity), aq. phosphate buffer pH 8, 28 °C, 200 rpm; (ii) *R. rhodochrous* IFO 15564 previously grown in the presence of DEPA, aq. phosphate buffer pH 8, 28 °C, 200 rpm; (iii) *R. rhodochrous* IFO 15564 (amidase activity) previously grown in the absence of DEPA, aq. phosphate buffer pH 8, 28 °C, 200 rpm.

Table 2. Molar percentages of recovered substances in *R. rhodochrous*-catalysed hydration of benzoylacetonitrile (**1d**)[a]

Row	A_{650}[b]	Reaction time (min)	*R. rhodochrous* grown in the absence of DEPA			*R. rhodochrous* grown in the presence of 15 mM DEPA		
			1d	**2d**	Σ[c]	**1d**	**2d**	Σ[c]
1	1.0	10	60	22	82	52	44	96
2	1.0	20	48	30	78	38	57	95
3	1.0	30	38	39	77	27	66	94
4	2.0	30	27	53	80	16	79	95
5	3.0	40	4	71	75	n.o.[d]	96	96
6	1.0	240	10	42	52	n.o.[d]	79	79

[a] **1d** (0.70 mmol, 102 mg) in EtOH (1.0 mL) was added to a suspension of *R. rhodochrous* in 0.10 M potassium phosphate buffer (100 mL, pH 8.0); rotary shaking (200 rpm) at 28 °C.
[b] Absorbance (optical density) of the bacterial suspension at 650 nm.
[c] Sum of percentages of **1d** and **2d**.
[d] Not observed by ^{1}H NMR.

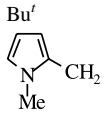
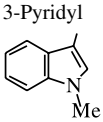
if added to the reaction mixture during the biotransformation,[17,18] which has the drawback of leading to an impure crude reaction product needing chromatographic purification. Thus, our observation is noteworthy that DEPA also works if added only during the growth of *R. rhodochrous*. This was our standard methodology, since it usually allows us to obtain crude β-ketoamides as NMR-pure compounds ready to use, for instance, as starting materials for further fungal bioreductions.

2.5. Preparative biotransformations

Scheme 1 shows the straightforward course of these biotransformations which, for preparative purposes, have to be stopped in the β-ketoamide **2** stage. Table 3 summarizes the preparation of β-ketoamides **2a**–**t**. Standard reaction conditions already were outlined in Section 2.4. A few cases deviated somewhat from the standard conditions and will be briefly discussed in the next paragraphs. Since typical bacterial nitrile hydrolyses were carried out in aqueous buffers containing ca. 0.1% [w(g)/v(mL)] of substrate,[13e] we usually started from 100–130 mg (0.60–0.80 mmol) of a β-ketonitrile **1** and a *R. rhodochrous* suspension in 100 mL of aqueous 0.10 M potassium phosphate buffer at pH 8.0 (A_{650}=3.0). The same results are roughly obtained if the pH varies in the range 6–8. For other details concerning the standard methodology, see Sections 3.3 and 3.4. Other non-standard processes will be discussed in Section 2.6.

All β-ketoamides yields lie in the good to excellent range 71–94%, except for the ketoamide **2q** (60%). Most of the crude products are pure β-ketoamides as their ^{1}H NMR spectra reveal. However, trace amounts of the β-ketonitriles **1** or the ketones **4** are also present in a few cases. Exceptionally, β-ketoamides **2i** and **2q** are contaminated

Table 3. β-Ketoamides **2** obtained by *R. rhodochrous*[a]-catalysed hydration[b] of β-ketonitriles **1**[c]

Compound	R^1	R^2	Reaction time (min)	Yield (%)[d]	Compound	R^1	R^2	Reaction time	Yield (%)[d]
2a	Pr	H	90	87	**2j**	4-Cl–C_6H_4	H	110 min	72
2b	Bu^t	H	25	90	**2k**[e]	3,4-$Cl_2C_6H_3$	H	60 min	71
2c		H	120	91	**2m**	2-Furyl	H	70 min	87
					2n	3-Furyl	H	40 min	87
					2p	2-Thienyl	H	75 min	89
					2q[f]	3-Pyridyl	H	9.5 h	60
2d	Ph	H	40	93	**2r**[g]		H	14 h	75
2e	3-Me–C_6H_4	H	60	93					
2f	4-Me–C_6H_4	H	60	88					
2g	4-MeO–C_6H_4	H	70	94					
2h	3-F_3C–C_6H_4	H	215	89	**2s**[h]	Me	Ph	23.5 h	79
2i[i]	3-Cl–C_6H_4	H	45	85	**2t**	Ph	Et	190 min	77

[a] Grown in sterilized medium M (see Section 3.3) plus 15 mM DEPA, except for **2s**.
[b] In a bacterial suspension (A_{650}=3.0, unless otherwise specified) in aq. 0.10 M phosphate buffer (100 mL), pH 8.0 (28 °C, 200 rpm).
[c] Except for **1i**,**k**,**r**, 100–130 mg (0.60–0.80 mmol) of starting material **1** in ethanol (1.0 mL) were added to the bacterial suspension.
[d] After flash column chromatography purification.
[e] Seventy four milligrams (0.35 mmol) of **1k** and 0.74 mL of ethanol were employed.
[f] The biotransformation was carried out with a bacterial absorbance of 6.7.
[g] Fifty milligrams (0.25 mmol) of **1r** and 0.50 mL of ethanol were employed; the biotransformation was carried out with a bacterial absorbance of 14.0.
[h] Bacteria were grown in sterilized medium N (see Section 3.3) plus 15 mM DEPA; the biotransformation was carried out with a bacterial absorbance of 11.6.
[i] 60 mg (0.33 mmol) of **1i** and 0.60 mL of ethanol were employed; the biotransformation was carried out in the presence of 5 mM DEPA.

by remarkable amounts of DEPA and ketone **4q**, respectively, due to their special reaction conditions (see below). Nevertheless, purification of all these compounds is easily and efficiently accomplished by flash column chromatography (see Section 3.4). For instance, from the crude product obtained after the biotransformation of **1q** (85:15 mixture **2q/4q**; crude yield, 63%), 60% yield of pure nicotinoylacetamide (**2q**) was reached.

All chlorinated β-ketonitriles (**1i**–**k**) and, especially, 3-(1-methylindol-3-yl)-3-oxopropanenitrile (**1r**) are only slightly soluble in the aqueous phosphate buffer, so that their bacterial hydration reactions are very slow. For instance, using **1r** as substrate under standard conditions, only ca. 17% of the corresponding amide **2r** was obtained after 5 days, together with ca. 40% of the remaining substrate (entrapped in the bacterial cake, from which its recovery is difficult) and ca. 40% of 3-acetyl-1-methylindole (**4r**).[31] Since much substrate was wasted, we decided to diminish its amount (only 50 mg, 0.25 mmol), and moreover, to increase the bacterial optical density to an A_{650} value of 14.0, in order to accelerate the biotransformation. Both experimental changes led to 80% crude yield of **2r** (75% after chromatographic purification), in spite of the long time (14 h) required for the disappearance of the substrate.

A good 71% yield of 2,3-dichlorobenzoylacetamide (**2k**) was obtained by merely starting from ca. half of the usual substrate amount. In the case of 3-chlorobenzoylacetamide (**2i**), the best yield (85%) was reached not only by using a reduced amount of **1i**, but also in the presence of 5 mM DEPA during the biotransformation. Under standard conditions, only 54 and 64% of **2k** and **2i**, respectively, could be obtained. In the case of 4-chlorobenzoylacetamide (**2j**), however, the standard methodology led to a good 74% yield, and no relevant improvements were observed by changing it.

The bacterial hydration of nicotinoylacetonitrile, **1q**, is also very slow. With the standard bacterial absorbance (A_{650}=3.0), only ca. 10% of nicotinoylacetamide, **2q**, was obtained after 6 h; most of the crude product was **1q**, together with a significant amount of gummy material. With the purpose of speeding up the bacterial hydration and decreasing the formation of impurities, we decided to increase the bacterial absorbance to an A_{650} value of 6.7. Thus, although some gummy material still appeared, we were able to isolate 60% of **2q** after 9.5 h.

Of α-substituted β-ketonitriles **1s** and **1t**, the latter offered no special problems, but the former failed as substrate under the standard conditions. In fact, after 24 h, TLC does not show noteworthy transformation of **1s**; after several days, some **2s** appeared (^{1}H NMR), but approximately half of the mass was lost and, moreover, the crude product was a complex mixture. After other failed attempts, we observed that the use of a somewhat more complex medium for growing the bacterium (medium N[28c] instead of the standard medium M, see Section 3.3) led to 79% yield of **2s** after a 23.5 h biotransformation. Despite this long reaction time, no β-ketoacid **3s** was observed. Moreover, the yield of **2s** was very similar (76%) after growing the bacterium in the absence of DEPA. These facts seem to prove the existence of some unfavourable steric and/or electronic interaction between the amide **2s** and the amidase.[32]

Finally, a β-ketonitrile not included in Table 3 also deserves some comments. 3-(2-Chlorophenyl)-3-oxopropanenitrile (**1**, R^1=2-Cl–C_6H_4, R^2=H) is an *ortho*-substituted aromatic substrate whose solubility in aqueous media is very low. Nonetheless, it was accepted by *R. rhodochrous* under standard conditions, TLC analysis showing a little amount of β-ketoamide, but not β-ketoacid, formed after 2 h. As the time elapses (10 h), most of the ketonitrile remains, a clear spot of ketoacid emerges, but only a trace of ketoamide is seen. Several variations were established in order to overcome the low solubility and/or to stop the process at the ketoamide stage (use of half amount of starting material, increase of the bacterial absorbance, presence of DEPA during biotransformation, change of the culture medium), some of them even simultaneously. However, the best yield in ketoamide was only 27%, this product being always accompanied by ketonitrile and 2-chloroacetophenone. Thus, we must conclude that this *ortho*-substitution negatively affects the nitrile hydratase activity of *R. rhodochrous*.

2.6. Biotransformations on a larger scale

Apart from the standard reactions used for synthetic purposes, it would be interesting to know to what extent the catalytic potential of the *R. rhodochrous* cells could be exploited. Thus, choosing benzoylacetonitrile (**1d**) as a reference substrate again, we initially carried out several experiments with the bacterium previously grown in the presence of DEPA and suspended (with a reference absorbance A_{650}=1.0) in 100 mL of the usual phosphate buffer. The cells contained in this suspension (resuspended in distilled water) gave ca. 90 mg of dry residue (cell dry weight, CDW) after evaporation at 120 °C.

Starting from 100 mg (0.69 mmol) of **1d**, a very good yield of **2d** was obtained after 2 h, just after **1d** disappeared (Table 4, entry 1). Doubling the amount of substrate, it was necessary to wait for 8 h until disappearance of the substrate (entry 2); moreover, the ketoamide yield strongly fell due to the residual amidase activity. Serious problems arose starting from 250 mg (1.72 mmol) of **1d** (entry 3). TLC monitoring showed that, at the most, only half of the substrate was transformed, approximately. A remarkable amount of β-ketoamide **2d** was formed in the beginning, but it was hydrolyzed to the corresponding acid and disappeared after 2 days. Thus, it seems that an excess of **1d** inhibits the

Table 4. Biotransformations of variable amounts of benzoylacetonitrile (**1d**) with *R. rhodochrous*[a] (A_{650}=1.0)

Entry	Mass of **1d**[b] (mg (mmol))	Reaction time (h)	Yield[c] of **2d** (%)
1	100 (0.69)	1.5	91
2	200 (1.38)	8	62
3	250 (1.72)	48[d]	—[d]

[a] Grown in the presence of 15 mM DEPA and suspended in 100 mL of 0.10 M potassium phosphate buffer, pH 8.0.
[b] 10 μL EtOH/mg **1d** were added to the reaction mixture.
[c] Determined by ^{1}H NMR of the crude reaction product.
[d] See text.

nitrile hydratase. Consequently, it can be estimated that the bacterial mass equivalent to ca. 100 mg of CDW does not accept more than about 150 mg (ca. 1 mmol) of **1d**. Our standard preparative substrate/bacterial mass ratio is notably lower in order to attain the best yields in the shortest reaction times.

With the data of the previous paragraph in mind, we resolved to carry out a series of larger scale experiments, all of them in such conditions that the inhibition by substrate of the nitrile hydratase was avoided. First we decided to recycle the cells in our standard conditions. After each reaction, cells were harvested by centrifugation, resuspended in fresh potassium phosphate buffer, and then exposed to a new amount of substrate **1d**. DEPA was present, as usual, during the growth of the bacterium, but not during the biotransformation. Using this methodology, yields of **2d** progressively diminished with the number of recyclings, so that in the fourth reaction only a very little amount of the substrate was transformed.

In view of the previous unsatisfactory results, we repeated the recycling experiments introducing a change, namely, adding 5 mM DEPA during the phase of reaction. The results are collected in Table 5. The four first ca. 1 h-reactions gave an excellent average yield of 93% of **2d** as the sole product. The fifth reaction is slightly worse, but, even so, it produces a very good yield. Altogether, almost 750 mg of **1d** were transformed by the cellular mass equivalent to 265 mg of CDW, i.e. ca. 1.95 mmol of **1d**/100 mg of CDW, which roughly doubles the previously estimated bioconversion efficiency for simple processes.

Another obvious method of avoiding the substrate inhibition would consist of the addition of the substrate in successive portions on a same bacterial suspension. To such an aim, we established three subroutines, according to the presence of DEPA only during the growth phase, only during the reaction phase, or during both phases. As expected, since this methodology implies a long time contact between the β-ketoamide **2d** and the amidase, the third subroutine was the best by far. To a bacterial suspension (A_{650}=4.0) in the usual phosphate buffer (100 mL) plus 5 mM DEPA, seven average 108 mg portions of **1d** were added in regular intervals of time during 8 h. The overall 755 mg (5.21 mmol) of **1d** were transformed into 672 mg (4.12 mmol, 79%) of **2d**, which was accompanied in the crude reaction product by 0.27 mmol (5%) of unchanged **1d**.[33] From these data, a bioconversion efficiency of 1.16 mmol of **1d**/100 mg of CDW can be deduced. As a corollary of these experiments, the nitrile hydratase and the amidase do not seem to be appreciably inhibited by a moderate excess of β-ketoamide **2d**.

Table 5. Biotransformations[a] (in the presence of DEPA) of benzoylacetonitrile (**1d**) with *R. rhodochrous*[b] recycling[c] (A_{650}=3.0)

Reaction no.	Reaction time (min)	Yield[d] of **2d** (%)
1	55	93
2	50	93
3	55	94
4	60	92
5	270	85

[a] 150 mg (1.03 mmol) of **1d** in 1.5 mL of EtOH were employed in each experiment.
[b] Grown in the presence of 15 mM DEPA and suspended in 100 mL of 0.10 M potassium phosphate buffer (pH 8.0) plus 5 mM DEPA.
[c] After each reaction, the bacterial cake was resuspended in 100 mL of fresh 0.10 M potassium phosphate buffer (pH 8.0) plus 5 mM DEPA, and more **1d**/EtOH was added.
[d] Determined by ^{1}H NMR of the crude reaction product.

From the point of view of the bioconversion efficiency, the recycling method (in the presence of DEPA) is better than that of feeding in portions, but also is more tedious, consumes more DEPA and requires more expensive column chromatography. Therefore, the selection of one or another will depend on the circumstances of each case.

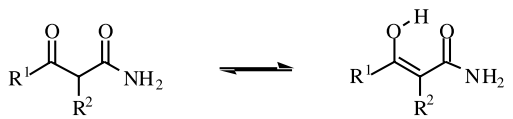
Finally, a further non-standard experiment is noteworthy. Using a 50 mL suspension of *R. rhodochrous* (A_{650}=11) in the usual potassium phosphate buffer plus 5 mM DEPA, 454 mg (3.13 mmol) of **1d** were converted in 485 mg (2.97 mmol, 92% yield) of β-ketoamide **2d** after 2 h. This result is not surprising, since the substrate/bacterial mass ratio is ca. one third lower than its previously estimated maximum value (1 mmol of substrate/100 mg CDW). However, this experiment represents an alternative, fast and efficient way of obtaining notable amounts of β-ketoamide from concentrated suspensions of the bacterium.

2.7. Keto–enol tautomerism in β-ketoamides 2a–t

Keto–enol tautomerism in β-ketoesters and β-diketones is a topic extensively studied from several points of view and by means of a variety of experimental methods.[34,35] This phenomenon, however, has been much less studied for β-ketoamides[34a,36] (Scheme 2). With a few exceptions,[37] it is usual to describe them only as keto forms,[10] despite some of them, in our hands, have shown to exist as a tautomeric mixture where the enol form was the major tautomer.[38] The apparent carelessness in this matter contrasts with the recent recognition of the β-ketoamides' enol forms as important structural elements of tetracyclines and piroxicams.[39]

In view of this situation, we show in Table 6 the percentages of enol forms in the keto–enol equilibria of β-ketoamides **2a–t** (Scheme 2). Data were obtained by ^{1}H NMR spectroscopy [in $CDCl_3$ for all of them and in CD_3OD and/or $(CD_3)_2CO$ for some of them], at 0.01–0.20 molar concentrations, after several days, in order to ensure that the equilibria were reached. As a general rule, however, no time dependence was observed in $CDCl_3$, whereas an almost imperceptible and a strong drop of the enol content in $(CD_3)_2CO$ and in CD_3OD, respectively, were noticed as the time elapses.

For β-ketoesters and β-diketones it is well established that the presence of electron-withdrawing substituents raises the



Scheme 2. Keto–enol tautomerism in β-ketoamides **2**.

Table 6. Percentages[a] of enol tautomers for β-ketoamides **2**[b] in three deuterated solvents[c]

Compound	R^1	$CDCl_3$	CD_3OD	$(CD_3)_2CO$	Compound	R^1	$CDCl_3$	CD_3OD	$(CD_3)_2CO$
2a	Pr	~3			**2j**	4-Cl–C_6H_4	17	23	60
2b	Bu^t	5	[d]	~40	**2k**	3,4-$Cl_2C_6H_3$	30		68
2c	1-(Methylpyrrol-2-yl)methyl	11			**2m**	2-Furyl	~3		
2d	Ph	12		55	**2n**	3-Furyl	~4	7	28
2e	3-Me–C_6H_4	9		51	**2p**	2-Thienyl	~2		16
2f	4-Me–C_6H_4	8	13	46	**2q**	3-Pyridyl	35	42	67
2g	4-MeO–C_6H_4	~2	6		**2r**	1-Methyl-indol-3-yl	0		
2h	3-F_3C–C_6H_4	30		68	**2s**	Me (R^2=Ph)	79	28	65
2i	3-Cl–C_6H_4	23		66	**2t**	Ph (R^2=Et)	0		0

[a] Determined at room temperature by ^1H NMR spectrometry after the equilibria were reached.
[b] Except for **2s**,**t**, R^2=H.
[c] Solute concentrations: 0.01–0.20 mol/L.
[d] This value clearly lies between 5 and 40%, although signal overlapping precludes its exact determination.

enol percentage,[34g,h] since electron withdrawal from the enol ring enhances the electrostatic contribution to the strength of the intramolecular hydrogen bond. Conversely, electron-releasing substituents decrease the enol content.

Such a tendency is clearly reflected in the enol percentages of monosubstituted 3-oxopropanamides **2a**–**r** when dissolved in deuteriochloroform (see Table 6). Since it is usually assumed that phenyl group is a weakly withdrawing one, the enol content of **2d** is somewhat higher than those of the alkyl substituted, weakly releasing **2a**,**b**.[40] If one considers the aryl substituted **2d**–**k** as a whole, it is evident that those bearing one or two further electron-withdrawing groups (**2h**–**k**) exhibit a higher enol percentage than the 'unsubstituted' **2d**, and this percentage increases with the withdrawing strength of the substituents (**2h**,**k**).[41] The opposite tendency is just observed for the aryl substituted **2e**–**g** bearing *meta*- and *para*-substituents that release electronic charge. The case of heterocyclic systems **2m**–**r** is also very illustrative. When the heterocycle is π-excessive, i.e. releases electronic charge (**2m**–**p**,**r**), the enol content is very low, especially for **2r**, whose indole ring is linked at its highly electron rich C-3 position. On the contrary, the presence of a π-deficient, highly electron-withdrawing pyridine ring (**2q**) dramatically increases the percentage of enol tautomer.

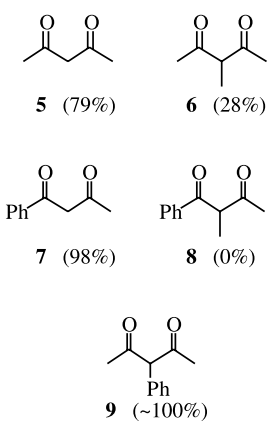


Figure 1. Enol percentages for several selected β-diketones (^1H NMR, $CDCl_3$). (Compounds **5**–**8**: Ref. 34g; Compound **9**: Ref. 43).

α,γ-Disubstituted 3-oxopropanamides **2s**,**t** should be commented independently. Alkyl substitution in the methylene group of β-diketones highly favours the keto tautomer, as shown by comparing diketones **5** and **6** and, especially **7** and **8** (Fig. 1). Although steric effects have been invoked to explain this fact,[34g] the matter is far from being clear, since similar steric hindrance are to be expected in the enol forms of β-diketones **8** and **9**, whose enol percentages differ as dramatically as shown in Figure 1. In any case, a parallelism between similarly substituted β-diketones **7** and **8** and β-ketoamides **2d** and **2t** accounts for the exclusive existence of the latter ketoamide as keto tautomer.[42] On the contrary, α-phenyl substituted β-ketoamide **2s** preponderantly exists as enol tautomer (79%), as it was to be expected after knowing the case of 3-phenylpentane-2,4-dione **9**.[43]

Finally, let us consider the influence of solvent polarity on the keto–enol equilibria of β-ketoamides **2**. For β-diketones and β-ketoesters, it is generally accepted that their tautomeric equilibria are shifted towards their less polar enol forms as the solvent polarity decreases.[35] Our data for β-ketoamides **2** (Table 6) concern three deuterated solvents, whose relative polarities depend on the method employed for evaluating them. For normal, non-deuterated solvents, polarities decrease in the series $MeOH > Me_2CO > CHCl_3$ when measured from spectroscopic data,[44] whereas they differ as $MeOH > CHCl_3 > Me_2CO$ if deduced from equilibria data[45] and from the solvent's anion-solvating and cation-solvating tendencies.[46]

Literature and Table 6 data show that the relationship between solvent polarity and keto–enol equilibria is a very complex problem. In fact, enol percentages deduced from ^1H NMR for pentane-2,4-dione increase as in the former series, i.e. $CD_3OD < (CD_3)_2CO < CDCl_3$,[47] whereas for ethyl acetoacetate they increase by the latter, namely, $CD_3OD < CDCl_3 < (CD_3)_2CO$.[34e] For their part, β-ketoamides **2** also behaves in their own way. Thus, all monosubstituted 3-oxopropanamides for which all three data are collected in Table 6 (**2b**,**f**,**j**,**n**,**q**) show increasing enol percentages in the series $CDCl_3 < CD_3OD \ll (CD_3)_2CO$, whereas the disubstituted **2s** behaves as pentane-2,4-dione. Thus, it seems that several factors, among which the high number of possible hydrogen bonds in N-unsubstituted β-ketoamides will probably play an important role, must be considered in depth in order to clarify this matter, but this study is beyond of the goals of this paper.

3. Experimental

3.1. General

Thin-layer chromatography was performed on precoated TLC plates of Merck silica gel $60F_{254}$, using potassium permanganate as developing reagent. For column chromatography, Merck silica gel 60 (particle size, 40–63 μm) was used. Melting points were taken using a Gallenkamp or a Stuart SMP3 apparatus and are uncorrected. Mass spectra were recorded on a Hewlett–Packard 5897 A (electron impact, 70 eV) and a Hewlett–Packard 1100 HPLC/MS (electrospray) instruments. The C, H, N analyses were performed on a Perkin–Elmer 2400 analyzer. Absorbances (optical densities) were measured at 650 nm on a Perkin–Elmer UV–Vis Lambda20 spectrometer. ^{1}H and ^{13}C NMR spectra were obtained with a Bruker AC-200 spectrometer (200.13 MHz for ^{1}H and 50.3 MHz for ^{13}C), using the δ scale (ppm) for chemical shifts; calibration was made on the corresponding solvent signal; ^{13}C NMR spectra were edited using DEPT techniques.

3.2. Starting materials 1

Substrates **1b**,**d**–**m**,**p**,**s** are commercially available (Aldrich, Avocado or Lancaster). Substrates **1a**,**c**,**n**,**q**,**r**,**t** were prepared by means of a modified previous method,[48] namely, by reacting acetonitrile (propionitrile for **2t**) with sodium amide (ca. −30 °C), and then with the respective R^1-CO_2Et (same temperature) until the ester disappeared.

3.3. Cultures of *R. rhodochrous* IFO 15564

R. rhodochrous IFO 15564 was maintained at 4 °C on Petri plates containing tryptone (10 g/L), yeast extract (5 g/L), NaCl (10 g/L) and agar (15 g/L), with pH adjusted at 7.0. Sub-culturing was performed every 3 months.

Except for the incubation of **1s**, the bacterium was grown in a culture medium M,[13e] whose components were heat-sterilized (115 °C, 20 min) in two separate groups. Group 1: glucose (15.0 g), yeast extract (1.0 g), KH_2PO_4 (0.50 g), K_2HPO_4 (0.50 g) and distilled water (950 mL), with pH adjusted at 7.2; group 2: ε-caprolactam (5.0 g), $FeSO_4{\cdot}7H_2O$ (0.50 g) and distilled water (50 mL). For the incubation of **1s**, the following medium N[28c] was employed: heat-sterilized (115 °C, 20 min): glucose (15.0 g), peptone (5.0 g), yeast extract (1.0 g), KH_2PO_4 (0.40 g), K_2HPO_4 (1.20 g), $MgSO_4{\cdot}7H_2O$ (0.50 g) and distilled water (950 mL), with pH adjusted at 7.2; filter-sterilized: ε-caprolactam (1.0 g), $FeSO_4{\cdot}7H_2O$ (0.30 g) and distilled water (50 mL).

A loop of solid culture of *R. rhodochrous* IFO 15564, from an agar plate, was sowed on 200–500 mL of sterilized medium M (medium N for the synthesis of **2s**), supplemented with 15 mM DEPA (from a 1.0 M reserve solution in methanol). After growing (rotary shaker, 200 rpm, 28 °C) for 36–42 h (medium M; final A_{650}=2–2.5) or for 60 h (medium N; final $A_{650}\cong$12.5), cells were harvested by centrifugation (5000 rpm, 3 min), washed with 0.10 M KH_2PO_4–K_2HPO_4 buffer pH 8.0 (20 mL), again collected by centrifugation and then resuspended in the same, fresh potassium phosphate buffer, adjusting the absorbance of the bacterial suspension to the value of 3.0, except for the preparation of **2q** (A_{650}=6.7), **2r** (A_{650}=14.0) and **2s** (A_{650}=11.6).

3.4. Standard preparations of β-ketoamides 2

To the appropriate bacterial suspension (100 mL) as described above, 0.60–0.80 mmol of the corresponding β-ketonitrile **1** (0.25–0.35 mmol in the cases of **1i**,**k**,**r**; vide infra) and ca. 1 mL of ethanol were added. In the case of **1i**, 5 mM DEPA (from a 1.0 M reserve solution in methanol) was also incorporated. Incubation (rotary shaker, 200 rpm, 28 °C) was carried out until the substrate disappeared (TLC monitoring, ethyl acetate as eluent; aliquots of 0.5 mL plus one drop of aqueous 1 M HCl, extracted with ethyl acetate), and then the cells were discarded by centrifugation (5000 rpm, 3 min). The supernatant, a slightly basic liquid, was continuously extracted with dichloromethane (8 h). [When concentrated bacterial suspensions were used (in the preparation of **2q**,**r**,**s**), the bacterial cake was washed with distilled water (20 mL), centrifuged again, and the supernatant liquid combined with the previous one before continuous extraction]. After drying the organic phase with anhydrous Na_2SO_4, low pressure elimination of solvent yielded the corresponding, essentially pure (except **2i**,**q**) β-ketoamide **2**. Further purification was accomplished by silica gel flash column chromatography [hexane–ethyl acetate as eluent, except for **2q** (see below)].

In this way, the following β-ketoamides **2** were obtained. To our knowledge, **2c**,**e**–**h**,**j**,**k**,**n**,**p**,**r** are new compounds. Complete NMR spectral data are included for all keto tautomers, whereas only selected resonances are shown for most enol tautomers. For signal assignments, Greek letters are used for the aliphatic chains, and numerical locants for the rings.

3.4.1. 3-Oxohexanamide (2a). From 90 mg (0.80 mmol) of **1a**/1.0 mL EtOH, 91 mg (0.69 mmol, 87%) of **2a** were obtained. Mp 80–81 °C (lit.,[49] 79–81 °C). ^{1}H NMR (keto, $CDCl_3$): 0.87 (t, $3H_\varepsilon$, J=7.4 Hz), 1.56 (sext., $2H_\delta$, J=7.4 Hz), 2.49 (t, $2H_\gamma$, J=7.4 Hz), 3.37 (s, $2H_\alpha$), 6.37 (broad s, 1NH), 7.06 (broad s, 1NH). ^{13}C NMR (keto, $CDCl_3$): 13.3 (C_ε), 16.6 (C_δ), 45.4 (C_γ), 48.7 (C_α), 168.5 (amide C), 206.3 (C_β). MS, *m*/*z* (%): 129 (M^+, 35), 114 (29), 112 (21), 101 (60), 86 (81), 71 (88), 59 (100).

3.4.2. 4,4-Dimethyl-3-oxopentanamide (2b). From 100 mg (0.80 mmol) of **1b**/1.0 mL EtOH, 103 mg (0.72 mmol, 90%) of **2b** were obtained. Mp 93–95 °C (lit., 95[50] and 83–84[7] °C). ^{1}H NMR (keto, $CDCl_3$): 1.15 (s, $9H_\delta$), 3.48 (s, $2H_\alpha$), 5.59 (broad s, 1NH), 7.06 (broad s, 1NH); [enol, $(CD_3)_2CO$]: 5.12 (s, $1H_\alpha$), 14.7 (s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 25.7 (C_δ), 43.3 (C_α), 45.0 (C_γ), 168.8 (amide C), 211.5 (C_β); (enol, $CDCl_3$): 27.3 (C_δ), 36.2 (C_γ), 85.3 (C_α), 174.7 (amide C), 184.8 (C_β). MS, *m*/*z* (%): 143 (M^+, 13), 115 (30), 111 (11), 86 (79), 59 (100), 57 (51).

3.4.3. 4-(1-Methylpyrrol-2-yl)methyl-3-oxobutanamide (2c). From 102 mg (0.63 mmol) of **1c**/1.0 mL EtOH, 103 mg (0.57 mmol, 91%) of **2c** (oil) were obtained. ^{1}H NMR (keto, $CDCl_3$): 3.42 (s, $2H_\alpha$), 3.47 (s, 3H, N–Me),

3.78 (s, 2H$_\gamma$), 6.0–6.15 (m, 2H, H-3 and H-4), 6.25 (broad s, 1NH), 6.60 (t, 1H, H-5, J=1.9 Hz), 6.76 (broad s, 1NH); (enol, $CDCl_3$): 4.60 (s, 1H$_\alpha$), 13.9 (s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 33.7 (N–Me), 41.8 (C$_\gamma$), 47.4 (C$_\alpha$), 107.2 and 108.7 (C-3 and C-4), 122.9 (C-5), 123.7 (C-2), 168.1 (amide C), 202.8 (C$_\beta$); (enol, $CDCl_3$): 89.5 (C$_\alpha$), 174.0 and 175.3 (amide C and C$_\beta$). MS, *m*/*z* (%): 180 (M$^+$, 58), 163 (7), 121 (20), 94 (100). Anal. (%) calcd for $C_9H_{12}N_2O_2$ (180.20): C, 59.99; H, 6.71; N, 15.55; found: C, 60.15; H, 6.58; N, 15.51.

3.4.4. 3-Oxo-3-phenylpropanamide (2d). From 101 mg (0.70 mmol) of **1d**/1.0 mL EtOH, 106 mg (0.65 mmol, 93%) of **2d** were obtained. Mp 111.5–113.5 °C (lit.,[10h] 110–111 °C). ^{1}H NMR (keto, $CDCl_3$): 3.98 (s, 2H$_\alpha$), 5.56 (broad s, 1NH), 7.13 (broad s, 1NH), 7.4–7.7 (m, 3H, H-3, H-4 and H-5), 7.95–8.1 (m, 2H, H-2 and H-6); (enol, $CDCl_3$): 5.54 (s, 1H$_\alpha$), 14.2 (s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 45.8 (C$_\alpha$), 129.2 and 129.5 (C-2, C-3, C-5 and C-6), 134.8 (C-4), 136.7 (C-1), 169.1 (amide C), 196.2 (C$_\beta$); (enol, $CDCl_3$): 88.1 (C$_\alpha$), 171.7 (amide C), 175.2 (C$_\beta$). MS, *m*/*z* (%): 163 (M$^+$, 13), 146 (9), 105 (100), 77 (46).

3.4.5. 3-(3-Methylphenyl)-3-oxopropanamide (2e). From 112 mg (0.70 mmol) of **1e**/1.0 mL EtOH, 116 mg (0.66 mmol, 93%) of **2e** were obtained. Mp 98.5–99.5 °C. ^{1}H NMR (keto, $CDCl_3$): 2.42 (s, 3H, Me), 3.96 (s, 2H$_\alpha$), 5.78 (broad s, 1NH), 7.16 (broad s, 1NH), 7.25–7.5 (m, 2H, H-4 and H-5), 7.75–7.9 (m, 2H, H-2 and H-6); (enol, $CDCl_3$): 5.55 (s, 1H$_\alpha$), 14.2 (s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 21.2 (Me), 45.0 (C$_\alpha$), 125.7 (C-6), 128.6 and 128.9 (C-2 and C-5), 134.8 (C-4), 136.0 (C-1), 138.6 (C-3), 168.4 (amide C), 195.7 (C$_\beta$); (enol, $CDCl_3$): 87.3 (C$_\alpha$). MS, *m*/*z* (%): 177 (M$^+$, 57), 160 (14), 120 (16), 119 (100), 91 (75). Anal. (%) calcd for $C_{10}H_{11}NO_2$ (177.20): C, 67.78; H, 6.26; N, 7.90; found: C, 67.90; H, 6.10; N, 7.69.

3.4.6. 3-(4-Methylphenyl)-3-oxopropanamide (2f). From 111 mg (0.70 mmol) of **1f**/1.0 mL EtOH, 109 mg (0.61 mmol, 88%) of **2f** were obtained. Mp 117–118 °C. ^{1}H NMR (keto, $CDCl_3$): 2.42 (s, 3H, Me), 3.94 (s, 2H$_\alpha$), 5.75 (broad s, 1NH), 7.16 (broad s, 1NH), 7.29 (d, 2H, H-3 and H-5, J=8.0 Hz), 7.89 (d, 2H, H-2 and H-6, J=8.0 Hz); (enol, $CDCl_3$): 5.52 (s, 1H$_\alpha$), 14.2 (s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 21.6 (Me), 44.9 (C$_\alpha$), 128.6 and 129.5 (C-2, C-3, C-5 and C-6), 133.5 (C-1), 145.1 (C-4), 168.5 (amide C), 195.1 (C$_\beta$); (enol, $CDCl_3$): 86.6 (C$_\alpha$). MS, *m*/*z*: 200.1 (M+Na)$^+$. Anal. (%) calcd for $C_{10}H_{11}NO_2$ (177.20): C, 67.78; H, 6.26; N, 7.90; found: C, 67.52; H, 6.26; N, 7.70.

3.4.7. 3-(4-Methoxyphenyl)-3-oxopropanamide (2g). From 112 mg (0.64 mmol) of **1g**/1.0 mL EtOH, 116 mg (0.60 mmol, 94%) of **2g** were obtained. Mp 145–146.5 °C. ^{1}H NMR (keto, $CDCl_3$): 3.89 (s, 3H, Me), 3.92 (s, 2H$_\alpha$), 5.56 (broad s, 1NH), 6.96 (d, 2H, H-3 and H-5, J=9.2 Hz), 7.18 (broad s, 1NH), 7.98 (d, 2H, H-2 and H-6, J=9.2 Hz); (enol, CD_3OD): 5.67 (s, 1H$_\alpha$). ^{13}C NMR (keto, $CDCl_3$): 44.6 (C$_\alpha$), 55.5 (Me), 114.0 (C-3 and C-5), 129.0 (C-1), 131.0 (C-2 and C-6), 164.3 (C-4), 168.3 (amide C), 194.0 (C$_\beta$). MS, *m*/*z* (%): 193 (M$^+$, 18), 149 (22), 135 (100), 97 (33), 91 (30), 83 (38), 77 (41), 71 (60), 69 (55), 57 (98), 55 (56). Anal. (%) calcd for $C_{10}H_{11}NO_3$ (193.20): C, 62.17; H, 5.74; N, 7.25; found: C, 62.34; H, 5.66; N, 7.14.

3.4.8. 3-Oxo-3-[(3-trifluoromethyl)phenyl]propanamide (2h). From 128 mg (0.60 mmol) of **1h**/1.0 mL EtOH, 123 mg (0.53 mmol, 88%) of **2h** were obtained. Mp 69–71 °C. ^{1}H NMR (keto, $CDCl_3$): 4.00 (s, 2H$_\alpha$), 5.93 (broad s, 1NH), 6.98 (broad s, 1NH), 7.65–7.8 (m, 1H, H-5), 7.85–7.95 (m, 1H, H-4), 8.2–8.4 (m, 2H, H-2 and H-6); (enol, $CDCl_3$): 5.61 (s, 1H$_\alpha$), 14.3 (broad s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 45.3 (C$_\alpha$), 122.7–136.5 (several aromatic and F_3C peaks), 167.6 (amide C), 194.1 (C$_\beta$); (enol, $CDCl_3$): 88.2 (C$_\alpha$), 169.4 (amide C), 173.9 (C$_\beta$). MS, *m*/*z* (%): 254 (M+Na)$^+$, 232 (M+H$^+$). Anal. (%) calcd for $C_{10}H_8F_3NO_2$ (231.17): C, 51.96; H, 3.49; N, 6.06; found: C, 51.70; H, 3.47; N, 5.78.

3.4.9. 3-(3-Chlorophenyl)-3-oxopropanamide (2i). From 60 mg (0.33 mmol) of **1i**/0.60 mL EtOH, 56 mg (0.28 mmol, 85%) of **2i** were obtained. Mp 136.5–138 °C (lit.,[10h] 135–137 °C). ^{1}H NMR (keto, $CDCl_3$): 3.96 (s, 2H$_\alpha$), 5.65 (broad s, 1NH), 7.02 (broad s, 1NH), 7.4–7.5 (m, 1H, H-5), 7.55–7.65 (m, 1H, H-4), 7.85–7.92 (m, 1H, H-6), 7.96–8.01 (m, 1H, H-2); (enol, $CDCl_3$): 5.55 (s, 1H$_\alpha$), 14.2 (s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 45.1 (C$_\alpha$), 126.7 (C-6), 128.5 (C-2), 130.2 (C-5), 134.0 (C-4), 135.3 (C-3), 137.5 (C-1), 167.4 (amide C), 194.3 (C$_\beta$); [enol, $(CD_3)_2CO$]: 89.9 (C$_\alpha$), 169.2 (amide C), 175.7 (C$_\beta$). MS, *m*/*z* (%): 222 (M+2+Na)$^+$, 220 (M+Na)$^+$. Anal. (%) calcd for $C_9H_8ClNO_2$ (197.62): C, 54.70; H, 4.08; N, 7.09; found: C, 54.73; H, 3.95; N, 6.93.

3.4.10. 3-(4-Chlorophenyl)-3-oxopropanamide (2j). From 128 mg (0.71 mmol) of **1j**/1.0 mL EtOH, 101 mg (0.51 mmol, 72%) of **2j** were obtained. Mp 143–145 °C (dec.). ^{1}H NMR (keto, $CDCl_3$): 3.94 (s, 2H$_\alpha$), 5.67 (broad s, 1NH), 6.98 (broad s, 1NH), 7.48 (d, 2H, H-3 and H-5, J=8.6 Hz), 7.94 (d, 2H, H-2 and H-6, J=8.6 Hz); (enol, $CDCl_3$): 5.53 (s, 1H$_\alpha$), 7.38 (d, 2H, H-3 and H-5, J=8.6 Hz), 7.69 (d, 2H, H-2 and H-6, J=8.6 Hz), 14.3 (broad s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 45.0 (C$_\alpha$), 129.2 (C-3 and C-5), 129.9 (C-2 and C-6), 134.3 (C-1), 140.8 (C-4), 167.6 (amide C), 194.3 (C$_\beta$); [enol, $(CD_3)_2CO$]: 88.6 (C$_\alpha$), 168.9 (amide C), 175.0 (C$_\beta$). MS, *m*/*z* (%): 199 [(M+2)$^+$, 4], 197 (M$^+$, 14), 141 (31), 139 (100), 111 (37). Anal. (%) calcd for $C_9H_8ClNO_2$ (197.62): C, 54.70; H, 4.08; N, 7.09; found: C,54.45; H, 4.17; N, 6.87.

3.4.11. 3-(3,4-Dichlorophenyl)-3-oxopropanamide (2k). From 74 mg (0.35 mmol) of **1k**/0.74 mL EtOH, 57 mg (0.25 mmol, 71%) of **2k** were obtained. Mp 114–115.5 °C. ^{1}H NMR (keto, $CDCl_3$): 3.94 (s, 2H$_\alpha$), 5.83 (broad s, 1NH), 6.93 (broad s, 1NH), 7.59 (d,1H, H-5, J=8.6 Hz), 7.83 (dd, 1H, H-6, J=2.3, 8.6 Hz), 8.09 (d, 1H, H-2, J=2.3 Hz); (enol, $CDCl_3$): 5.53 (s, 1H$_\alpha$), 14.2 (broad s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 45.2 (C$_\alpha$), 127.6–138.9 (six aromatic signals), 167.2 (amide C), 193.2 (C$_\beta$); (enol, $CDCl_3$): 88.0 (C$_\alpha$), 168.9 (amide C), 173.7 (C$_\beta$). MS, *m*/*z* (%): 272 (M+2+K)$^+$, 270 (M+K)$^+$, 256 (M+2+Na)$^+$, 254 (M+Na)$^+$, 232 (M+H)$^+$. Anal. (%) calcd for $C_9H_7Cl_2NO_2$ (232.06): C, 46.58; H, 3.04; N, 6.04; found: C, 46.80; H, 2.89; N, 5.91.

3.4.12. 3-(2-Furyl)-3-oxopropanamide (2m). From 103 mg (0.76 mmol) of **1m**/1.0 mL EtOH, 102 mg (0.66 mmol, 87%) of **2m** were obtained. Mp 142.5–144 °C (lit.,[51]

159 °C). ^{1}H NMR (keto, $CDCl_3$): 3.85 (s, $2H_\alpha$), 5.60 (broad s, 1NH), 7.10 (broad s, 1NH), 6.61 (dd, 1H, H-4, J=1.6, 3.7 Hz), 7.35 (d, 1H, H-3, J=3.7 Hz), 7.67 (d, 1H, H-5, J=1.6 Hz); (enol, $CDCl_3$): 5.52 (s, $1H_\alpha$). ^{13}C NMR (keto, $CDCl_3$): 44.5 (C_α), 112.9 (C-4), 119.6 (C-3), 147.9 (C-5), 151.8 (C-2), 167.4 (amide C), 183.7 (C_β). MS, *m*/*z* (%): 153 (M^+, 67), 110 (28), 97 (19), 95 (100), 71 (23), 57 (31).

3.4.13. 3-(3-Furyl)-3-oxopropanamide (2n). From 108 mg (0.80 mmol) of **1n**/1.0 mL EtOH, 106 mg (0.70 mmol, 87%) of **2n** were obtained. Mp 105.5–107 °C. ^{1}H NMR (keto, $CDCl_3$): 3.75 (s, $2H_\alpha$), 5.61 (broad s, 1NH), 6.80 (dd, 1H, H-4, J=0.8, 2.0 Hz), 7.10 (broad s, 1NH), 7.48 (t, 1H, H-5, J=1.6 Hz), 8.15 (broad s, 1H, H-2); [enol, $(CD_3)_2CO$]: 5.54 (s, $1H_\alpha$), 6.65 (d, 1H, H-4, J=1.0 Hz), 7.61 (t, 1H, H-5, J=1.6 Hz), 7.96 (broad s, 1H, H-2). ^{13}C NMR (keto, CD_3OD): 109.3 (C-4), 128.8 (C-3), 146.1 (C-5), 150.8 (C-2), 171.7 (amide C), 190.5 (C_β); C_α signal is overlapped with the solvent signal. MS, *m*/*z* (%): 153 (M^+, 14), 111 (18), 97 (14), 95 (100), 71 (14), 57 (17). Anal. (%) calcd for $C_7H_7NO_3$ (153.14): C, 54.90; H, 4.61; N, 9.15; found: C, 54.64; H, 4.78; N, 8.97.

3.4.14. 3-Oxo-3-(2-thienyl)propanamide (2p). From 100 mg (0.66 mmol) of **1p**/1.0 mL EtOH, 100 mg (0.59 mmol, 89%) of **2p** were obtained. Mp 125.5–127 °C. ^{1}H NMR (keto, $CDCl_3$): 3.92 (s, $2H_\alpha$), 5.57 (broad s, 1NH), 7.18 (dd, 1H, H-4, J=3.9, 5.1 Hz), 7.20 (broad s, 1NH), 7.76 (dd, 1H, H-5 or H-3, J=1.2, 5.1 Hz), 7.83 (dd, 1H, H-3 or H-5, J=1.2, 3.9 Hz); [enol, $(CD_3)_2CO$]: 5.72 (s, $1H_\alpha$), 7.13 (dd, 1H, H-4, J=3.9, 4.9 Hz), 7.55 (m, 1H, H-5 or H-3), 7.63 (broad d, 1H, H-3 or H-5, J=4.9 Hz). ^{13}C NMR [keto, $(CD_3)_2CO$]: 48.1 (C_α), 129.6 (C-4), 135.0 and 135.9 (C-5 and C-3), 145.3 (C-2), 168.7 (amide C), 188.4 (C_β); [enol, $(CD_3)_2CO$]: 88.0 (C_α). MS, *m*/*z* (%): 169 (M^+, 27), 111 (100). Anal. (%) calcd for $C_7H_7NO_2S$ (169.20): C, 49.69; H, 4.17; N, 8.28; found: C, 49.78; H, 4.12; N, 8.30.

3.4.15. 3-Oxo-3-(3-pyridyl)propanamide (2q). Due to the special acid–base properties of this product, the work-up was slightly different from the standard protocol. After the bioconversion [103 mg (0.71 mmol) of **1p**/1.0 mL EtOH], the supernatant liquid (ca. 100 mL) was concentrated in vacuo until ca. 10 mL, and then extracted with ethyl acetate–methanol 10:1 (5×10 mL), without washing. Usual drying and elimination of solvents led to a 85:15 crude mixture of **2q**: 3-acetylpyridine (**4q**), finally purified by flash column chromatography (eluent, chloroform–hexane–diethyl ether–methanol 3:1:1:0.7), to yield 69 mg (0.42 mmol, 60%) of **2q**. Mp 110.5–112 °C (lit.,[34a] 102–104 °C). ^{1}H NMR (keto, $CDCl_3$): 4.00 (s, $2H_\alpha$), 5.64 (broad s, 1NH), 6.94 (broad s, 1NH), 7.45 (dd, 1H, H-5, J=4.7, 8.2 Hz), 8.29 (dt, 1H, H-4, J=8.2, 2.0 Hz), 8.84 (dd, 1H, H-6, J=1.6, 4.7 Hz), 9.21 (d, 1H, H-2, J=2.3 Hz); (enol, $CDCl_3$): 5.60 (s, $1H_\alpha$), 7.37 (dd, 1H, H-5, J=4.7, 8.0 Hz), 8.06 (dt, 1H, H-4, J=7.8, 2.0 Hz), 8.66 (dd, 1H, H-6, J=1.6, 4.7 Hz), 8.96 (d, 1H, H-2, J=2.0 Hz), 14.2 (s, 1H, OH). ^{13}C NMR (keto, CD_3OD): 125.4 (C-5), 133.6 (C-3), 137.9 (C-4), 150.4 (C-6), 154.2 (C-2), 171.7 (amide C), 194.8 (C_β); C_α signal is overlapped with the solvent signal. MS, *m*/*z* (%): 164 (M^+, 52), 147 (91), 106 (100), 78 (66), 51 (20).

3.4.16. 3-(1-Methylindol-3-yl)-3-oxopropanamide (2r). From 50 mg (0.25 mmol) of **1r**/1.0 mL EtOH, 41 mg (0.19 mmol, 75%) of **2r** were obtained. Mp 179.5–181.5 °C. ^{1}H NMR (keto, $CDCl_3$): 3.81 (s, $2H_\alpha$), 3.88 (s, 1H, N–Me), 5.48 (broad s, 1NH), 7.2–7.5 (m, $3H_{Ar}$+1NH), 7.87 (s, 1H, H-2), 8.3–8.45 (m, 1H, H-4). ^{13}C NMR (keto, $CDCl_3$): 33.9 (N–Me), 46.7 (C_α), 110.0 (C-7), 116.1 (C-3), 122.5, 123.3 and 124.0 (C-4, C-5 and C-6), 126.3 (C-3a), 137.2 (C-2), 126.3 (C-7a), 169.1 (amide C), 189.4 (C_β). MS, *m*/*z* (%): 216 (M^+, 26), 158 (100), 130 (12). Anal. (%) calcd for $C_{12}H_{12}N_2O_2$ (216.24): C, 66.65; H, 5.59; N, 12.96; found: C, 66.39; H, 5.55; N, 12.79.

3.4.17. 3-Oxo-2-phenylbutanamide (2s). From 101 mg (0.64 mmol) of **1s**/1.0 mL EtOH, 89 mg (0.50 mmol, 79%) of **2s** were obtained. Mp 122.5–124.5 °C (lit.,[10h] 124–126 °C). ^{1}H NMR (keto, CD_3OD):[52] 2.22 (s, 3H, C_γ), 4.81 (s, $1H_\alpha$), 7.3–7.5 (m, $5H_{Ar}$); (enol, $CDCl_3$): 1.78 (s, 3H, C_γ), 5.07 (broad s, 1NH), 5.30 (broad s, 1NH), 7.2–7.5 (m, $5H_{Ar}$), 14.6 (s, 1H, OH). ^{13}C NMR (keto, $CDCl_3$): 29.8 (C_γ), 65.0 (C_α), 170.6 (amide C), 204.8 (C_β); aromatic carbon nuclei masked by the aromatic signals due to the major (79%) enol tautomer; (enol, $CDCl_3$): 19.7 (C_γ), 104.0 (C_α), 129.1, 131.2, 133.5 and 135.5 (aromatic carbon atoms), 172.4 (amide C), 174.6 (C_β). MS, *m*/*z* (%): 177 (M^+, 22), 160 (56), 135 (38), 118 (100), 105 (18), 90 (39), 77 (24).

3.4.18. 2-Benzoylbutanamide (2t). From 116 mg (0.67 mmol) of **1t**/1.0 mL EtOH, 99 mg (0.52 mmol, 77%) of **2t** were obtained. Mp 154–156 °C (lit.,[53] 153–153.5 °C). ^{1}H NMR (keto, $CDCl_3$): 1.00 (t, $3H_\delta$, J=7.4 Hz), 1.9–2.04 (m, $2H_\gamma$), 4.29 (t, $1H_\alpha$, J=7.2 Hz), 5.46 (broad s, 1NH), 6.59 (broad s, 1NH), 7.4–7.7 (m, 3H, H-3, H-4 and H-5), 8.02 (d, 2H, H-2 and H-6, J=7.0 Hz). ^{13}C NMR (keto, CD_3OD): 13.4 (C_δ), 25.2 (C_γ), 58.8 (C_α), 130.5 and 130.8 (C-2, C-3, C-5 and C-6), 135.6 (C-4), 139.0 (C-1), 175.7 (amide C), 199.0 (C_β). MS, *m*/*z* (%): 191 (M^+, 2), 163 (5), 122 (13), 105 (100), 77 (48).

3.5. Determination of keto–enol ratio in β-ketoamides 2

For each compound and solvent, comparison of integral values (^{1}H NMR) was made between those cleaner, non-overlapped signals corresponding to both tautomers. Thus, the most useful signals to this end with samples in $CDCl_3$ were those of H_α protons (**2a**–**e**,**h**,**k**,**m**,**p**), followed by H-2 and H-6 protons (*ortho* to keto or enol group: **2d**,**f**,**g**,**j**) and H-2 protons (**2i**,**n**); for **2s**, the signals due to H_γ protons were selected, whereas an average of integral values of H-2, H-4 and H-6 protons was taken for **2q**.

Using CD_3OD as solvent, H_α signals disappeared as a result of H/D interchange, in such a way that the determination was impossible in several cases (for instance, **2d**,**h**). However, keto–enol percentages were easily deduced from all aromatic or heterocyclic protons (**2f**,**j**,**n**,**p**), from *tert*-butyl protons (**2b**), from H_γ protons (**2s**) and from H-2, H-4 and H-6 protons (**2q**).

β-Ketoamides **2** showed a very slow H_α/D interchange when dissolved in $(CD_3)_2CO$ (except in the case of **2f**). For such a reason, H_α protons could be selected for a freshly prepared sample of **2b**, since the enol percentages remain

almost invariable with time in this solvent. H-2 and H-6 protons were useful for measurements in **2d**–**f**,**j**. The signals selected for the remaining four compounds were as follows: H-2 (**2n**); H-3 and H-5 (**2p**); H-2, H-4 and H-6 (**2q**); H_γ (**2s**).

Acknowledgements

Financial supports of this work by Principado de Asturias (Project GE-EXP01-03) and MCYT (Project PPQ-2001-2683) are gratefully acknowledged.

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32. When grown in this new culture medium N, *R. rhodochrous* has shown to develop amidase activity[28c].
33. We assume that the remaining 16% includes the formed β-ketoacid **3d** and small losses during the work-up.

34. See for example: (a) Bunting, J. W.; Kanter, J. P. *J. Am. Chem. Soc.* **1993**, *115*, 11705–11715. (b) Yamasaki, K.; Kajimoto, O. *Chem. Phys. Lett.* **1990**, *172*, 271–274. (c) Kallury, K. R.; Krull, U. J.; Thompson, M. *J. Org. Chem.* **1988**, *53*, 1320–1322. (d) Zheglova, D. Kh.; Aleksiev, A. A.; Kmetska, G. G.; Petrov, G. N.; Kol'tsov, A. I. *J. Org. Chem. USSR* **1988**, *24*, 1641–1643. (e) Mills, S. G.; Beak, P. *J. Org. Chem.* **1985**, *50*, 1216–1224. (f) Billman, J. H.; Sojka, S.; Taylor, P. R. *J. Chem. Soc., Perkin Trans. 2* **1972**, 2034–2035. (g) Allen, G.; Dwek, R. A. *J. Chem. Soc. (B)* **1966**, 161–163. (h) Burdett, J. L.; Rogers, M. T. *J. Am. Chem. Soc.* **1964**, *86*, 2105–2109.
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36. (a) Hynes, M. J.; Clarke, E. M. *J. Chem. Soc., Perkin Trans. 1* **1994**, 901–904. (b) Wengenroth, H.; Meier, H. *Chem. Ber.* **1990**, *123*, 1403–1409. (c) Naoum, M. M.; Saad, G. R. *J. Solution Chem.* **1988**, *17*, 67–76. (d) Pashkevich, K. I.; Saloutin, V. I.; Fomin, A. N.; Rudaya, M. N.; Egorova, L. C. *Izv. Akad. Nauk. SSSR, Ser. Khim.* **1986**, 1586–1592. (e) Dudek, G. O.; Volpp, G. P. *J. Org. Chem.* **1965**, *30*, 50–54.
37. Apart from papers with tautomeric orientation,[36] see for instance Ref. 10d.
38. A clear example is that of β-ketoamide **2s**. Our ^{1}H NMR spectrum ($CDCl_3$) shows that it is a 21:79 keto–enol mixture; however, it has been described as only keto form from ^{1}H NMR ($CDCl_3$).[10g]
39. Hansen, P. E.; Duus, F.; Bolvig, S.; Jagodzinski, T. S. *J. Mol. Struct.* **1996**, *378*, 45–59.
40. Although **2c** is formally alkyl substituted, the pyrrole ring can substantially alter its electronic effects.
41. Two β-ketoamides analogous to **2**, bearing strong electron-withdrawing substituents [R^1=F_3C, R^2=H; and R^1=$H(CF_2)_4$, R^2=H], have been described to exist only as enol forms in $CDCl_3$[36d].
42. When **2d** and **2t** bear an additional *N*-phenyl substituent, concordant enol percentages of 20 and 0%, respectively, were measured in chloroform using UV techniques[36c].
43. Okuro, K.; Furuune, M.; Miura, M.; Nomura, M. *J. Org. Chem.* **1993**, *58*, 7606–7607.
44. See Ref. 35, pp 360 and 365.
45. See Ref. 35, p 113.
46. See Ref. 35, p 402.
47. Data from Ref. 34e and own [$(CD_3)_2CO$].
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52. When freshly dissolved in CD_3OD, the keto–enol ratio is 95:5, and virtually no H_α/D interchange is observed. In $CDCl_3$ (keto): 2.24 (s, H_γ), 4.66 (s, H_α), 5.75 (broad s, 1NH), 6.90 (broad s, 1NH); H_{Ar} masked by the aromatic signals due to the major (79%) enol tautomer.
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