Pergamon

Tetrahedron, Vol. 53, No. 18, pp. 6421-6432, 1997
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0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)00301-3

Fungal and Bacterial Regioselective Hydroxylation of Pyrimidine Heterocycles

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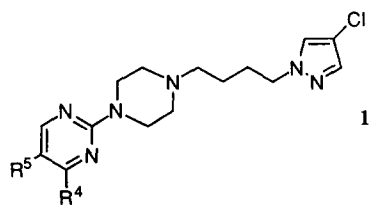
Abstract. The bacterium *Rhodococcus erythropolis* is employed to hydroxylate the anxiolytic lesopitron, and this bacterium, together with *Agrobacterium* sp. and the fungus *Beauveria bassiana*, are used to extend the field of hydroxylation of heteroaromatic compounds to a series of unexplored pyrimidines. Of all the substrates investigated, only the carbamate **11a** is regioselectively hydroxylated by *B. bassiana* at the C-5 position of the pyrimidine ring; in contrast, the bacteria are able to regioselectively oxidize, when free, the C-2 and/or C-4 positions of the pyrimidine moiety of all the substrates **1a** - **12a**, up to a maximum of two oxidations. © 1997 Elsevier Science Ltd.

INTRODUCTION

Apart from classical barbiturates, the pyrimidine ring is present in several pharmaceuticals, such as the hypertensive minoxidil or the anxiolytic buspirone,[1] as well as in other antimicrobial and antitumour agents.[2] Our interest in the new, non-benzodiazepine anxiolytic lesopitron[3] (**1a**, figure 1), prompted us to investigate the chemical synthesis of some of its human metabolites (*e.g.*, its hydroxy derivative at pyrimidine ring C-5 position, **1c**), or even other putative hydroxy metabolites (*e.g.*, **1b**). However, this task proved to be a very difficult one.[4] Moreover, direct regioselective hydroxylation of the pyrimidine ring by merely chemical methodologies is also another notoriously difficult goal,[5] as is in general the regioselective hydroxylation of aromatic heterocyclic compounds.



1 a, $R^4 = R^5 = H$
b, $R^4 = OH$, $R^5 = H$
c, $R^4 = H$, $R^5 = OH$

Figure 1. Lesopitron (**1a**) and two of its metabolites.

In view of this, and taking into account that hydroxylation is often the first step in the degradation of aromatic compounds by microorganisms in the environment, we considered microbial hydroxylation as an alternative way to attain our objectives. In the field of the microbial monohydroxylation of aromatics,[6] the areas

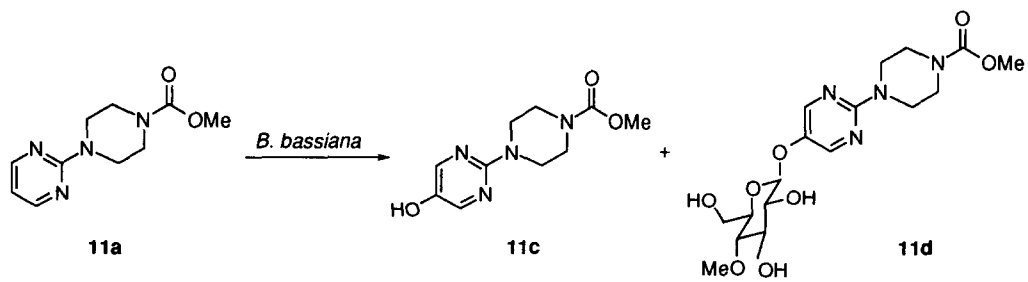
of carbo- and heterocyclic aromatic compounds have to be distinguished. Among the cell cultures used to monohydroxylate aromatic carbocycles,[7] those of the fungus *Beauveria bassiana*[8] could be the most promising in order to achieve our goals, since they are capable of functionalizing *para* positions to nitrogen substituents,[9,10] a feature which can be observed at the pyrimidine moiety of the metabolite **1c**. There is a limited number of examples of microbial monohydroxylation of heteroaromatic compounds, but these are very recent (since 1992),[11] are practically restricted to two rings (substituted pyridines[12] and pyrazines[13]), and always bear a relation to the pharmaceutical or agrochemical significance of the resulting metabolites. In addition, only bacteria,[14] which invariably oxidize a carbon atom adjacent to a nitrogen one, have been used for this purpose.

Although quinazoline (benzo[*d*]pyrimidine) has been submitted to microbial *vicinal cis*-dihydroxylation,[15] it is quite surprising that, to our knowledge, there are no reports on microbial monohydroxylation of pyrimidine rings.[16] Thus, together with our first purpose of preparing some metabolites of lesopitron, the objective of this work is also to widen the scope of microbial monohydroxylation to other related pyrimidine systems.

RESULTS AND DISCUSSION

In accordance with the literature and after preliminary experimental work, three microorganisms were selected for this study: the fungus *Beauveria bassiana* ATCC 7159 and the bacteria *Agrobacterium* sp. DSM 6136 and *Rhodococcus erythropolis* DSM 6138.

All our attempts to hydroxylate lesopitron (**1a**) or the simple pyrimidine rings **2a** - **7a** (figure 2) with *Beauveria bassiana* ATCC 7159, under a varied set of experimental conditions, were unsuccessful. Nevertheless, it has been reported that the hydroxylation of both aromatic[9c] and non-aromatic[17] systems with this fungus seems to be strongly dependent on the existence in the substrate of an electron-rich center (amide, sulfonamide, carbamate, etc.), which acts as an anchoring group to the enzymic active site.[18] Consequently, we conventionally prepared several carbamates and amides derived from 2-aminopyrimidine (substrates **8a** and **9a**) and from 2-(piperazin-1-yl)pyrimidine (substrates **10a** - **12a**), and exposed them to cultures of *B. bassiana*. However, only 2-[4-(methoxycarbonyl)piperazin-1-yl]pyrimidine (**11a**) was hydroxylated at the C-5 pyrimidine position, yielding 11% of **11c**, together with 8% of the corresponding 4-*O*-methyl-ß-D-glucopyranosyl derivative **11d** (scheme 1). The formation of similar phenol glucosides by *B. bassiana*, probably as part of its solubilization and detoxication mechanisms, has also been previously observed.[9ac,10,19]



Scheme 1

It has been described that *N*-phenylcarbamates, Ph-NH-CO-OR, are *para*-hydroxylated by *Beauveria bassiana*,[9c] the presence of conjugation between aromatic and carbamate moieties seeming to be a prerequisite for the process. This observation is in contrast with our results. In fact, the only hydroxylated pyrimidine substrate

was **11a**, in which no conjugation between pyrimidine and carbamate regions is present, whilst neither of the conjugated systems **8a** nor **9a** suffered oxidation in our hands. These results reflect the state of empiricism nowadays governing this field of microbial transformations.

Despite the fact that only one substrate is transformed by *B. bassiana*, it is noteworthy that, to the best of our knowledge, this is the first example of *fungal* hydroxylation of a non-fused aromatic heterocycle. Moreover, the obtained metabolite **11c** can be regarded as a potential precursor of the human lesopitron metabolite **1c** by means of chemical methods.[4]

It is now widely believed that the enzyme used by *B. bassiana* to hydroxylate aromatic carbocycles is the haem-dependent monooxygenase cytochrome P-450$_{cam}$.[9c,20] However, as the field of fungal hydroxylation of heteroaromatic compounds remains virtually unexplored, it seems advisable to corroborate this hypothesis in our case.

1-Aminobenzotriazole is a benzyne precursor that strongly inactivates cytochrome P-450$_{cam}$, both in microsomal preparations[21] and in microbial cultures,[22] due to the formation of an adduct between benzyne and two of the vicinal nitrogens of the cytochrome's porphyrin ring. We have exposed the substrate **11a** to a culture of *B. bassiana* in the presence of 1-aminobenzotriazole, and the substrate was recovered unchanged after the usual four days of the standard experiment. Thus, it appears that cytochrome P-450$_{cam}$ is also responsible for the monohydroxylation of pyrimidine ring of **11a** described here.

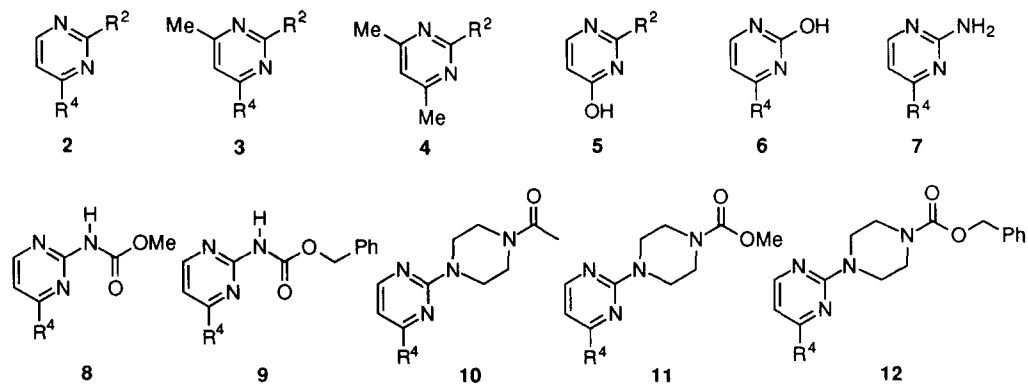


Figure 2. Pyrimidine substrates (**a**; R^2 and/or R^4 = H) and their bacterial metabolites (**b**; R^2 and/or R^4 = OH). Metabolites **2b**, **5b**, and **6b** are identical (uracil). For simplicity, they are represented as their less stable hydroxy tautomer (see text and figure 3).

At this point, we decided to expose the substrates **1a** - **12a** to the bacteria *Agrobacterium* sp. and *Rhodococcus erythropolis* (see figures 1 and 2, and table 1). The more simple ones, **2a** - **7a**, proved to be substrates only for *Agrobacterium* sp. (except 2-aminopyrimidine, **7a**, which was also hydroxylated by *R. erythropolis*; entries 7 and 8), while the others, more complex (**1a** and **8a** - **12a**), were transformed only by *R. erythropolis*, with a new exception (**8a**; entries 9 and 10) discussed below. Most reactions were carried out until disappearance of the substrate, as judged by TLC monitoring and confirmed by the NMR spectra of the crude reaction products, and usually led to very high yields of the corresponding metabolites, **1b** - **12b**.

As a summary of our synthetic results, and in relation to the literature reports, the following observations

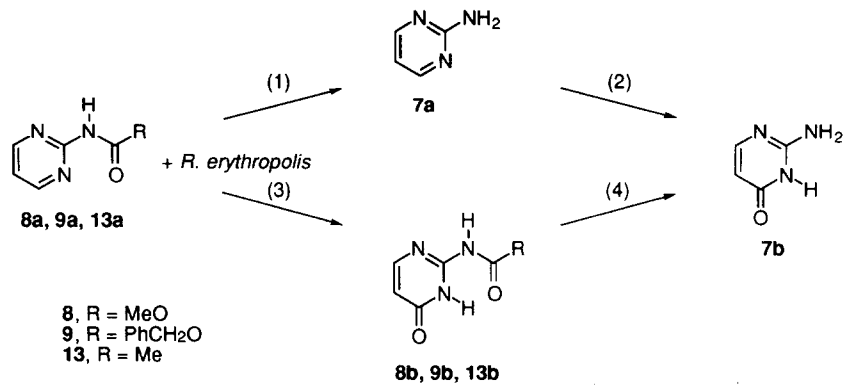
can be made. (1) As with other bacterial hydroxylations of aromatic heterocycles,[12,13] our reactions occur at carbon atoms adjacent to nitrogen ones. (2) Substrate structure permiting, up to a maximum of two oxidations take place, which can probably be related to the high stability and wide biological spread of the uracil-type systems. (3) *Microbial* hydroxylation at a position flanked by two nitrogen atoms (substrates **2a** - **5a**) was previously unknown; however, exposure of quinazoline[23] or pteridine systems[24] (both bearing a pyrimidine ring) to mammalian molybdenum-containing *isolated enzymes* resulted in di- or trihydroxylation patterns, which include functionalization on the C-2 position of pyrimidine ring.

Table 1. Bacterial transformations[a] of the pyrimidine-type substrates **1a** - **12a**

Entry	Substrate	Bacterium[b]	Reaction time (h)	Metabolite	Yield[c] (%)
1	**1a**[d]	R	96	**1b**	84
2	**2a**	A	23	**2b**[e]	77
3	**3a**	A	30	**3b**[f]	78
4	**4a**	A	48	**4b**	70
5	**5a**	A	15	**5b** = **2b**[e]	78
6	**6a**[d]	A	15	**6b** = **2b**[e]	85
7	**7a**	A	48	**7b**[g]	85
8	**7a**	R	48	**7b**[g]	74
9	**8a**	R	22	**8b** + **7a**[h]	92[i]
10	**8a**[j]	A	96	**8b**	35
11	**9a**	R	16	**9b**	58[k]
12	**10a**[l]	R	120	**10b**	65
13	**11a**	R	86	**11b**	98
14	**12a**	R	16	**12b**	80

[a] Biotransformations are stopped at 100% conversion of the substrates, unless otherwise specified. [b] A = *Agrobacterium* sp. DSM 6136; R = *Rhodococcus erythropolis* DSM 6138. [c] Isolated yield, after recrystallization or flash column cromatography. [d] Used as hydrochloride. [e] Uracil. [f] 6-Methyluracil. [g] Isocytosine. [h] *ca.* 4:1 mixture with some **7b**. [i] Overall yield. [j] 38% of conversion (see text). [k] Low yield due to the simultaneous formation of **7a** and **7b**. [l] 81% of conversion.

Besides its hydroxylase activity, *R. erythropolis* DSM 6138 also shows carbamate hydrolase behaviour, which partly complicates the biotransformations of the carbamates **8a** and **9a**.[25] Thus, depending on reaction time, 2-(methoxycarbonylamino)pyrimidine (**8a**) is converted into several mixtures of its hydroxy metabolite (**8b**), 2-aminopyrimidine (**7a**), and the hydroxy metabolite of the latter (**7b**) (*e. g.*, entry 9, table 1). The formation pathways of all these metabolites are indicated in scheme 2 (R = MeO).[26] Formation of **7a** [hydrolysis step (1)] and **8b** [hydroxylation step (3)] was monitored by TLC; the hydroxylation step (2) is consistent with entry 8, while the hydrolysis step (4) was stated by means of an independent experiment, in which a sample of **8b** was fully hydrolyzed by *R. erythropolis* after 52 hours. Since we were unable to isolate the desired **8b** from the reaction mixtures of the biotransformations of **8a** with *R. erythropolis*, a pure specimen of **8b** had to be obtained, although in low yield, by exposing **8a** to *Agrobacterium* sp. (entry 10).



Scheme 2

Similar double-pathway problems, but without separation difficulties, arise with 2-(benzyloxycarbonyl-amino)pyrimidine (**9a**; scheme 2, R = $PhCH_2O$; table 1, entry 11). These hydrolysis problems are not observed with carbamates **11a** and **12a**, probably due to the poorer ability of the piperazine ring as leaving group.

Pyrimidine substrates bearing amide function seem to be badly hydroxylated by these bacteria. Thus, the only amide included in this study (**10a**) is so slowly metabolized that its bacterial transformation had to be stopped before reaching its total conversion. Moreover, 2-(acetylamino)pyrimidine (**13a**) has not been included here as substrate since, in the presence of *R. erythropolis*, it is converted into the metabolite **7b**, following the double-pathway processes outlined in scheme 2 (R = Me). In this case, the hydrolysis steps are notably faster than the hydroxylation ones. Since hydrolysis is not observed when a blank reaction of **13a** is carried out, this is another example in which the DSM 6138 strain of *R. erythropolis* shows hydrolase (amidase) activity.[27]

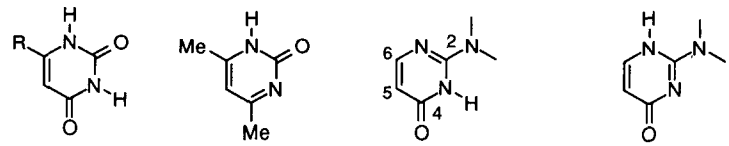
As was indicated above, isolated molybdoenzymes are responsible for the hydroxylation of aromatic heterocycles at positions adjacent to nitrogen atoms.[23,24] Several substances are known to inhibit the activity of such a type of enzymes. Thus, the cyanide anion is a general inhibitor for them, since it removes the sulphur from the molybdenum cofactor as thiocyanate.[28] Allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine) and menadione (2-methyl-1,4-naphtoquinone) are more specific inhibitors which strongly inactivate xanthine oxidase and aldehyde oxidase, respectively.[23,24b]

In our case, when pyrimidine (**2a**) is exposed to a culture of *Agrobacterium* sp. in the presence of cyanide anion, only trace amounts of uracil (**2b**) are formed after 14 hours, while a parallel standard experiment is almost finished within this time. Similarly, hydroxylation of 2-aminopyrimidine (**7a**) with *R. erythropolis* is inhibited by menadione, since in the presence of this substance only trace amounts of the metabolite **7b** are formed within the 48 hours of the standard experiment. In contrast, allopurinol does not inhibit this hydroxylation. As expected, then, some type of molybdoenzyme seems to be involved in the bacterial oxidations described here.

As mentioned in the caption of figure 2, the bacterial metabolites **1b** - **12b** have been represented as hydroxy tautomeric forms only for reasons of simplicity. However, it is well known that they exist predominantly as oxo (lactame) or dioxo (dilactame) tautomers,[29] and, in fact, spectral data (including variable temperature ^{1}H-NMR experiments carried out in deuteriochloroform for **9b** and **11b**) reveal for each compound the sole presence of one of these two structures, which have been represented initially in figure 3. Nevertheless, from the spectra of the generic 2-amino-4-pyrimidones (**1b** and **7b** - **12b**) it is difficult to discern between the above (3*H*)- and the other hypothetical (1*H*)-isomer, **14**.

On the other hand, isocytosine itself (**7b**) is known to be a 1:1 mixture of both tautomers in the crystalline

state, while 6-methylisocytosine may appear as the (3*H*)-tautomer or as a mixture of both of them in different solvents.[30] Thus, in order to clarify the structures of our metabolites **1b** and **7b** - **12b**, we have carried out two dimensional CH heteronuclear correlation experiments (HMBC[31]) on **11b** and **9b** in deuteriochloroform, as examples of metabolites with and without the piperazine moiety, respectively. The experiment with **11b** shows that the N-*H* proton gives only two cross peaks with *C-4* (^2J) and *C-5* (^3J), thus supporting the hypothesis of the 2-aminopyrimidin-4(3*H*)-one tautomers.[32] This hypothesis also agrees with the reported greater stability of the isocytosine (3*H*)-structure compared to its (1*H*)-counterpart, as calculated from spectroscopic data[33] and from the so-called aromaticity index.[34]



2b, 3b, 5b, 6b **4b** **1b, 7b - 12b** **14**

Figure 3. Actual (3*H*)-tautomers of the microbial metabolites **1b** - **12b** and hypothetical, non-observed (1*H*)-tautomer **14**.

EXPERIMENTAL

General

For column chromatography, Merck silica gel 60 (particle size, 40 - 63 μm) was used. Melting points were taken using a Gallenkamp apparatus and are uncorrected. Mass spectra were recorded on a Hewlett-Packard 5897 A spectrometer. Elemental analyses were performed on a Perkin-Elmer analyzer 2400. Optical densities were measured on a Shimadzu UV-160A spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were obtained with tetramethylsilane as internal standard, using a Bruker AC-200 spectrometer (200.13 MHz for ^{1}H and 50.3 MHz for ^{13}C), unless otherwise specified. ^{13}C-NMR spectra were edited using DEPT techniques. HMBC experiments were carried out using standard Bruker software. The assignments of the ^{1}H- and ^{13}C-NMR spectra of compounds **9b** and **11b** were deduced by trivial analysis of the HMBC spectra (Bruker AMX-400 spectrometer; 400.13 MHz for the ^{1}H and 100.61 MHz for the ^{13}C nuclei). ^{13}C-NMR spectra of the other related compounds could be reasonably assigned taking into account the almost invariable chemical shifts observed for the homologous carbon atoms and, particularly, for the quaternary and piperazine ones.

Substrates

Substrates **2a** -**7a** were purchased from Aldrich; lesopitron (**1a**) was supplied by Laboratorios Dr. Esteve, S.A. Carbamates **8a** and **9a** were conventionally prepared from 2-aminopyrimidine (**7a**) and methyl or benzyl chloroformate, respectively. Carbamates **11a** and **12a** were obtained in a similar way, but starting from 2-(piperazin-1-yl)pyrimidine. Amide **10a** was prepared from 2-(piperazin-1-yl)pyrimidine and acetyl chloride. Substrates **8a** - **12a** are previously unknown compounds.

2-(Methoxycarbonylamino)pyrimidine, **8a**. M.p.: 133.4-133.9 °C. ^{1}H-NMR ($CDCl_3$), δ (ppm): 3.88 (s, 3H, CH_3), 7.05 (t, 1H, *H-5*), 8.69 (d, 2H, *H-4* and *H-6*), *ca.* 10.4 (br. s, 1H, N*H*). ^{13}C-NMR ($CDCl_3$), δ

(ppm): 52.5 (CH_3), 115.7 (*C-5*), 152.2 (*C-2*), 157.5 (carbamate *C*=O), 158.3 (*C-4* and *C-6*). Anal. (%) calc. for $C_6H_7N_3O_2$ (153.14): C, 47.06; H, 4.61; N, 27.44; found: C, 46.82; H, 4.73; N, 27.72.

2-(Benzyloxycarbonylamino)pyrimidine, **9a**. M.p.: 166.5-168.0 °C. ^{1}H-NMR ($CDCl_3$), δ (ppm): 5.28 (s, 2H, O-CH_2-Ph), 6.80 (t, 1H, *H-5*, J_{54} = 4.8 Hz), 7.3-7.5 (m, $5H_{arom.}$), 8.50 (d, 2H, *H-4* and *H-6*, J_{45} = 4.8 Hz), *ca.* 10.4 (br. s, 1H, N*H*). ^{13}C-NMR ($CDCl_3$), δ (ppm): 67.3 (CH_2), 115.6 (*C-5*), 128.0 (d), 128.4 (d), 128.7 (d), 135.4 (s), 151.5 (*C-2*), 157.6 (carbamate *C*=O), 158.2 (*C-4* and *C-6*). Anal. (%) calc. for $C_{12}H_{11}N_3O_2$ (229.24): C, 62.87; H, 4.84; N, 18.33; found: C, 62.60; H, 4.96; N, 18.06.

2-(4-Ethanoylpiperazin-1-yl)pyrimidine, **10a**. M.p.: 91.8-92.8 °C. ^{1}H-NMR ($CDCl_3$), δ (ppm) [Bruker AC-300 spectrometer]: 2.09 (s, 3H, CH_3), 3.47 (dist. t, 2H, piperazine CH_2), 3.64 (dist. t, 2H, piperazine CH_2), 3.77 (m, 4H, 2 piperazine CH_2), 6.48 (t, 1H, *H-5*, J_{54} = 4.7 Hz), 8.26 (d, 2H, *H-4* and *H-6*, J_{45} = 4.7 Hz). ^{13}C-NMR ($CDCl_3$), δ (ppm) [Bruker AC-300 spectrometer]: 21.3 (CH_3), 41.0, 43.2, 43.5 and 45.8 (4 piperazine CH_2), 110.2 (*C-5*), 157.6 (*C-4* and *C-6*), 161.2 (*C-2*), 169.0 (amide *C*=O). Anal. (%) calc. for $C_{10}H_{14}N_4O$ (206.25): C, 58.24; H, 6.84; N, 27.17; found: C, 57.93; H, 6.89; N, 27.00.

2-[4-(Methoxycarbonyl)piperazin-1-yl]pyrimidine, **11a**. M.p.: 87.4-87.9 °C. ^{1}H-NMR ($CDCl_3$), δ (ppm): 3.50 (dist. t, 4H, 2 piperazine CH_2), 3.69 (s, 3H, CH_3), 3.78 (dist. t, 4H, 2 piperazine CH_2), 6.48 (t, 1H, *H-5*, J_{54} = 4.7 Hz), 8.27 (d, 2H, *H-4* and *H-6*, J_{45} = 4.7 Hz). ^{13}C-NMR ($CDCl_3$), δ (ppm): 43.3 (2 piperazine CH_2), 43.4 (2 piperazine CH_2), 52.5 (CH_3), 110.1 (*C-5*), 155.8 (carbamate *C*=O), 157.6 (*C-4*, and *C-6*), 161.3 (*C-2*). Anal. (%) calc. for $C_{10}H_{14}N_4O_2$ (222.25): C, 54.04; H, 6.35; N, 25.21; found: C, 53.93; H, 6.47; N, 24.98.

2-[4-(Benzyloxycarbonyl)piperazin-1-yl]pyrimidine, **12a**. M.p.: 70.8-72.1 °C. ^{1}H-NMR ($CDCl_3$), δ (ppm): 3.56 (dist. t, 4H, 2 piperazine CH_2), 3.81 (dist. t, 4H, 2 piperazine CH_2), 5.16 (s, 2H, O-CH_2-Ph), 6.50 (t, 1H, *H-5*), 7.35 (m, $5H_{arom.}$), 8.30 (d, 2H, *H-4* and *H-6*). ^{13}C-NMR ($CDCl_3$), δ (ppm): 43.2 (2 piperazine CH_2), 43.5 (2 piperazine CH_2), 67.1 (O-CH_2-Ph), 110.1 (*C-5*), 127.8 (d), 127.9 (d), 128.4 (d), 136.4 (s), 155.1 (carbamate *C*=O), 157.6 (*C-4* and *C-6*), 161.3 (*C-2*). Anal. (%) calc. for $C_{16}H_{18}N_4O_2$ (298.35): C, 64.41; H, 6.08; N, 18.78; found: C, 64.11; H, 6.07; N, 18.51.

Cultures of Beauveria bassiana *ATCC 7159*

The growing medium is composed by corn steep liquor (Sigma, C-4648; 20 g) and D-glucose (10 g) in 1 L of distilled water, adjusted to pH 4.85 with 3 N aqueous sodium hydroxide. The sterilized medium (300 mL, in a 1 L Erlenmeyer flask) is inoculated by a 48 hours-old vegetative culture (10 mL) and incubated (rotary shaker, 200 rpm, 28 °C) during 36 - 40 hours. The mycelium is then harvested by centrifugation (5500 rpm, 12 min) and resuspended in 100 mL of the supernatant medium.

Biotransformation of 2-[4-(methoxycarbonyl)piperazin-1-yl]pyrimidine, **11a**, *with* B. bassiana *ATCC 7159*

An ethanolic solution of **11a** (120 mg/1,2 mL) is added to the above fungal suspension (100 mL, in a 1 L Erlenmeyer flask) and incubation is continued during 96 hours. The mycelium is then centrifuged (as above), washed with distilled water, and the combined aqueous phases continuously extracted (24 hours) with dichloromethane. After drying with anhydrous Na_2SO_4, the organic solvent is distilled under reduced pressure. The crude residue is subsequently purified by flash column chromatography (eluent, chloroform : hexane : diethyl ether : methanol 3 : 2 : 1 : 0.5) to yield 13.5 mg (11%) of 2-[4-(methoxycarbonyl)piperazin-1-yl]pyrimidin-5-ol (**11c**). After a change in the eluent composition (3 : 1 : 1 : 0.7), 10 mg (8%) of **11d** (the 4-*O*-methyl-ß-D-glucopyranosyl derivative of **11c**) is also isolated.

2-[4-(Methoxycarbonyl)piperazin-1-yl]pyrimidin-5-ol, **11c**. M.p.: 192-196 °C (dec.). ^{1}H-NMR (CD_3OD), δ (ppm): 3.68-3.78 (m, 4H, 2 piperazine CH_2), 3.80-3.91 (m, 4H, 2 piperazine CH_2), 3.95 (s, 3H, CH_3), 8.22 (s, 2H, *H-4* and *H-6*). ^{13}C-NMR (CD_3OD), δ (ppm): 44.9 (2 piperazine CH_2), 45.9 (2 piperazine CH_2), 53.7 (CH_3), 146.1 (*C-5*), 146.8 (*C-4* and *C-6*), 158.0 (s), 158.6 (s). MS, m/z (%): 238 (88, M+), 223 (19, M – CH_3), 150 (29), 137 (45), 124 (100). Anal. (%) calc. for $C_{10}H_{14}N_4O_3$ (238.25): C, 50.41; H, 5.92; N, 23.52; found: C, 50.19; H, 6.19; N, 23.61.

2-[4-(Methoxycarbonyl)piperazin-1-yl]-5-(4-O-methyl-ß-D-glucopyranosyloxy)pyrimidine, **11d**. M.p.: 165.2-166.6 °C. ^{1}H-NMR ($CDCl_3$), δ (ppm): 3.34-3.94 (m, $8H_{piper.}$ + $4H_{gluc.}$), 3.62 (superimp. s, 3H, gluc. CH_3-O), 3.75 (superimp. s, 3H, carbamate CH_3-O), 4.67 (d, 1H, anomeric *CH*), 8.20 (s, 2H, *C-4* and *C-6*). ^{13}C-NMR (DMSO-d_6), δ (ppm): 43.1 (2 piperazine CH_2), 43.7 (2 piperazine CH_2), 52.5 (carbamate *C*=O), 59.8 (gluc. CH_3-O), 60.3 (gluc. *C-6*), 73.3, 75.7, 76.2, 79.1 (4 gluc. *C*H-O), 101.8 (anomeric *C*H), 144.1 (*C-5*), 147.9 (*C-4* and *C-6*), 157.7 (s), 158.0 (s). MS, m/z (%): 414 (M+, 0.53), 383 (M – CH_3O, 0.65), 238 (97), 150 (37), 124 (100). Anal. (%) calc. for $C_{17}H_{26}N_4O_8$ (414.42): C, 49.27; H, 6.32; N, 13.52; found: C, 49.02; H, 6.39; N, 13.74.

Cultures of Agrobacterium *sp. DSM 6136*

The composition of the growing medium is as follows (concentration in mg/L).[13b] Heat sterilized: $(NH_4)_2SO_4$ (2000), Na_2HPO_4 (2000), KH_2PO_4 (1000), NaCl (3000), $MgCl_2{\cdot}6H_2O$ (400), $CaCl_2{\cdot}2H_2O$ (14.5), $FeCl_3{\cdot}6H_2O$ (0.8), $ZnSO_4{\cdot}7H_2O$ (0.10), $MnCl_2{\cdot}4H_2O$ (0.09), H_3BO_3 (0.30), $CoCl_2{\cdot}6H_2O$ (0.20), $CuCl_2{\cdot}2H_2O$ (0.01), $NiCl_2{\cdot}6H_2O$ (0.02), $Na_2MoO_4{\cdot}2H_2O$ (0.03), $EDTANa_2{\cdot}2H_2O$ (0.005), $FeSO_4{\cdot}7H_2O$ (0.002) (pH of the solution adjusted to 7.0); filter sterilized: pyridoxal hydrochloride (0.01), riboflavin (0.005), nicotinamide (0.005), thiamin hydrochloride (0.002), biotin (0.002), pantothenic acid (0.005), *p*-aminobenzoic acid (0.005), folic acid (0.002), vitamin B_{12} (0.005); this medium is supplemented with pyrazine (1000) as carbon, nitrogen and energy source.

The medium (600 mL, in a 2 L Erlenmeyer flask) is inoculated with a 72 hours-old culture (50 mL) and incubated (rotary shaker, 200 rpm, 28 °C) until an optical density of *ca.* 1.0 at 650 nm. Then the cells are centrifuged (5500 rpm, 12 min), resuspended in fresh medium (*without* pyrazine) and adjusted to an optical density of *ca.* 10 (650 nm).

Biotransformations with Agrobacterium *sp. DSM 6136*

Substrates **2a** - **8a** (140 mg) are added (**6a** as hydrochloride; **8a** dissolved in 400-800 µL of *N,N*-dimethylformamide) to the latter bacterial suspension (70 mL, in a 250 mL Erlenmeyer flask) and incubation is continued until disappearance (TLC monitoring; same eluents as for *B. bassiana* transformation) of the substrate (in the case of **8a**, until a conversion of *ca.* 40%). The cells are then centrifuged (as above), washed with distilled water, and the combined aqueous phases continuously extracted (24 hours) with ethyl acetate. After drying with anhydrous Na_2SO_4, the organic solvent is distilled under reduced pressure and the crude product recrystallized, except in the case of **8b**, which is purified by flash column chromatography (eluent, dichloromethane : ethyl acetate 2 : 1 to recover the unreacted **8a**; then, diethyl ether : acetone : hexane 2 : 2 : 1 to isolate **8b**). In this way, the following metabolites are isolated:

Pyrimidine-2,4(1H,3H)-dione (uracil),[35a] **2b**, **5b** or **6b**.

6-Methylpyrimidine-2,4(1H,3H)-dione (6-methyluracil),[35b] **3b**.

4,6-Dimethylpyrimidin-2(1H)-one,[35c] **4b**.

2-Aminopyrimidin-4(3H)-one (isocytosine),[35d] **7b**.

2-(Methoxycarbonylamino)pyrimidin-4(3H)-one, **8b**. M.p.: 219-224 °C (dec.) ^{1}H-NMR (DMSO-d_6), δ (ppm): 3.84 (s, 3H, CH_3), 6.05 (d, 1H, *H-5*, J_{56} = 6.9 Hz), 7.82 (d, 1H, *H-6*, J_{65} = 6.9 Hz), *ca.* 11.4-11.8 (br. s. 2 N*H*). ^{13}C-NMR (DMSO-d_6), δ (ppm): 52.9 (*C*H$_3$), 107.9 (*C-5*), 150.8 (*C-6*), 151.8 (*C-2*), 156.4 (carbamate *C*=O), 161.8 (*C-4*). MS, *m/z*: 169 (M$^+$, 100%), 137 (25%). Anal. (%) calc. for $C_6H_7N_3O_3$: C, 42.61; H, 4.17; N, 24.84; found: C, 42.64; H, 4.27; N, 24.56.

Melting points and spectroscopic data of the well known compounds **2b** - **7b** agree with those of authentical samples purchased from Aldrich.

Cultures of Rhodococcus erythropolis *DSM 6138*

The composition of the growing medium[13a] is the same as for *Agrobacterium* sp. DSM 6136, except that the concentration of $CoCl_2 \cdot 6H_2O$ is only 0.01 mg/L and $CuCl_2 \cdot 2H_2O$ is not present; the medium is supplemented with 1000 mg/L of 2,5-dimethylpyrazine instead of pyrazine. The protocol previous to the biotransformations is similar to that of *Agrobacterium* sp. (final resuspension *in the absence* of 2,5-dimethylpyrazine).

Biotransformations with Rhodococcus erythropolis *DSM 6138*

Substrates **1a** and **9a** - **12a** (140 mg) are added as follows: **1a** as hydrochloride; **9a** dissolved in 400-800 µL of ethanol; **10a** - **12a** dissolved in 400-800 µL of *N,N*-dimethylformamide. Only for **10a** the incubation process is stopped before total conversion (*ca.* 80%). Continuous extraction is performed with ethyl acetate or dichloromethane, according to the case. Purification of crude product is as follows: **1b**, recrystallization from hexane : chloroform *ca.* 10 : 1; **9b** - **12b**, flash column chromatography [same eluent as for *B. bassiana* transformations, except for **10b** (diethyl ether : methanol 1 : 1)]. For the rest of the experimental method, see biotransformations with *Agrobacterium* sp. The following metabolites are isolated:

2-{4-[4-(4-Chloro-1H-pyrazol-1-yl)butyl]piperazin-1-yl}pyrimidin-4(3H)-one, **1b**. M.p.: 137.1-138.9 °C. ^{1}H-NMR (CDCl_3), δ (ppm): 1.46 (dist. quint., 2H), 1.86 (dist. quint., 2H), 2.34 (t, 2H, *J* = 7.0 Hz), 2.45 (br. s, 4H), 3.72 (br. s, 4H), 4.07 (t, 2H, *J* = 7.0 Hz), 5.71 (d, 1H, pyrimidine *H-5*, J_{56} = 6.4 Hz), 7.36 (s, 1H, pyrazol ring), 7.39 (s, 1H, pyrazol ring), 7.72 (d, 1H, pyrimidine *H-6*, J_{65} = 6.4 Hz), *ca.* 11.7 (br. s, N*H*). ^{13}C-NMR (CDCl_3), δ (ppm): 23.4 (t), 27.8 (t), 44.2 (t), 52.4 (2 t), 57.3 (t), 102.0 (pyrimidine *C-5*), 109.3 (pyrazol *C*-Cl), 126.7 (pyrazol *C-5*), 137.2 (pyrazol *C-3*), 154.3 (pyrimidine *C-2*), 156.9 (pyrimidine *C-6*), 165.8 (pyrimidine *C-4*). Anal. (%) calc. for $C_{15}H_{21}ClN_6O$ (336.83): C, 53.49; H, 6.28; N, 24.95; found: C, 53.27; H, 6.10; N, 25.12.

2-(Benzyloxycarbonylamino)pyrimidin-4(3H)-one, **9b**. M.p.: 173.9-175.6 °C. ^{1}H-NMR (CDCl_3), δ (ppm): 5.25 (s, 2H, O-CH_2-Ph), 5.89 (d, 1H, *H-5*, J_{56} = 7.0 Hz), 7.36 (d, 1H, *H-6*), 7.4 (superimp. br. s, 5H$_{arom.}$), 11.9-12.3 (br. s., 2 N*H*). ^{13}C-NMR (CDCl_3), δ (ppm): 68.8 (O-*C*H$_2$-Ph), 109.5 (*C-5*), 128.5 (d), 128.8 (d), 129.1 (d), 134.1 (s), 151.8 (*C-4*), 152.5 (*C-6*), 154.1 (*C-2*), 160.5 (carbamate *C*=O). MS, *m/z*: 245 (M$^+$, 8%), 91 (100%). Anal. (%) calc. for $C_{12}H_{11}N_3O_3$ (245.24): C, 58.77; H, 4.52; N, 17.13; found: C,

58.89; H, 4.32; N, 17.40.

2-(4-Ethanoylpiperazin-1-yl)pyrimidin-4(3H)-one, **10b**. M.p.: 208-212 (dec.) ^{1}H-NMR ($CDCl_3$), δ (ppm): 2.14 (s, 3H, CH_3), *ca.* 3.5-4.0 (m, 8H, 4 piperazine CH_2), 5.77 (d, 1H, *H-5*, J_{56} = 6.4 Hz), 7.76 (d, 1H, *H-6*, J_{65} = 6.4 Hz), *ca.* 12.2 (br. s, N*H*). ^{13}C-NMR ($CDCl_3$), δ (ppm): 21.3 (CH_3), 40.8 (piperazine CH_2), 44.4 (2 piperazine CH_2), 45.6 (piperazine CH_2), 102.9 (*C-5*), 154.4 (*C-2*), 157.0 (*C-6*), 165.9 (*C-4*), 169.2 (amide *C*=O). MS, *m/z*: 222 (M+, 42%), 124 (100%), 150 (65%), 95 (14%). Anal. (%) calc. for $C_{10}H_{14}N_4O_2$ (222.25): C, 54.04; H, 6.35; N, 25.21; found: C, 54.23; H, 6.09; N, 25.17.

2-[4-(Methoxycarbonyl)piperazin-1-yl]pyrimidin-4(3H)-one, **11b**. M.p: 220-222 °C. ^{1}H-NMR ($CDCl_3$), δ (ppm): 3.59 (m, 4H, 2 piperazine CH_2), 3.74 (s, 3 H, CH_3), 3.74 (superimp. m, 4H, 2 piperazine CH_2), 5.76 (d, 1H, *H-5*, J_{56} = 6.3 Hz), 7.76 (d, 1H, *H-6*, J_{65} = 6.3 Hz), *ca.* 12.3 (br. s, 1H, N*H*). ^{13}C-NMR ($CDCl_3$), δ (ppm): 43.2 (2 piperazine CH_2-N-CO), 44.3 (t, 2 piperazine CH_2-N-pyrim.), 52.8 (CH_3), 102.8 (*C-5*), 154.4 (*C-2*), 155.7 (carbamate *C*=O), 157.0 (*C-6*), 165.9 (*C-4*). MS, *m/z*: 238 (M+, 17%), 150 (26%), 124 (100%), 95 (10%). Anal. (%) calc. for $C_{10}H_{14}N_4O_3$ (238.25): C, 50.41; H, 5.92; N, 23.52; found: C, 50.28; H, 6.00; N, 23.51.

2-[4-(Benzyloxycarbonyl)piperazin-1-yl]pyrimidin-4(3H)-one, **12b**. M.p.: 145.0-146.5 °C. ^{1}H-NMR ($CDCl_3$), δ (ppm): 3.63 (m, 4H, 2 piperazine CH_2), 3.77 (m, 4H, 2 piperazine CH_2), 5.18 (s, 2H, O-CH_2Ph), 5.78 (d, 1H, *H-5*, J_{56} = 6.3 Hz), 7.38 (br. s, $5H_{arom.}$), 7.77 (d, 1H, *H-6*, J_{65} = 6.3 Hz), *ca.* 12.2 (br. s, N*H*). ^{13}C-NMR ($CDCl_3$), δ (ppm): 43.2 (2 piperazine CH_2-N-CO), 44.3 (2 piperazine CH_2-N-pyrim.), 67.4 (O-CH_2-Ph), 102.8 (*C-5*), 128.0 (d), 128.1 (d), 128.4 (d), 136.2 (s), 154.4 (*C-2*), 155.1 (carbamate *C*=O), 157.0 (*C-6*), 166.0 (*C-4*). MS, *m/z*: 314 (M+, 21%), 223 (17%), 150 (16%), 124 (93%), 91 (100%). Anal. (%) calc. for $C_{16}H_{18}N_4O_3$ (314.35): C, 61.14; H, 5.77; N, 17.82; found: C, 61.21; H, 5.84; N, 17.56.

Inhibition experiments

The reaction conditions of these experiments are identical to the standard ones, except in the addition of the corresponding inhibitor. All the experiments are positive (nothing or only trace amounts of the corresponding metabolite are detected), except with the use of allopurinol.

Incubation of B. bassiana *with* **11a** *and 1-aminobenzotriazole.* A solution of 33 mg of 1-aminobenzotriazole in 500 μL of ethanol is added, to give a 2.5 mM concentration of the inhibitor in the reaction medium.

Incubation of Agrobacterium *sp. with* **2a** *and cyanide anion.* A diluted aqueous solution of potassium cyanide is added in such a way that the concentration of cyanide anion in the reaction medium is 5 nM.

Incubation of R. erythropolis *with* **7a** *and menadione.* Menadione (2.1 mg dissolved in 420 μL of ethanol; 0.80 mM concentration in the reaction medium) is preincubated during 5 minutes prior to addition of substrate.

Incubation of R. erythropolis *with* **7a** *and allopurinol.* Allopurinol is also preincubated during 5 minutes prior to addition of substrate, two concentrations of the former being employed: 0.50 mM (0.82 mg in 164 μL of ethanol) and 2.0 mM (4.1 mg in 820 μL of ethanol).

ACKNOWLEDGEMENTS

Financial support of this work by Comisión de Investigación Científica y Técnica (CICYT; project BIO 95-0687) is gratefully acknowledged. M.Q. thanks the FICYT (Asturias) for a predoctoral scholarship. We also thank Servicios Comunes de Investigación de la Universidad de Oviedo for the measurements of 2D and variable temperature NMR spectra.

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(*Received in UK* 4 *February* 1997; *revised* 17 *March* 1997; *accepted* 20 *March* 1997)