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Microbial hydroxylation of benzoxazoles containing fluorine atoms in the aromatic ring—tracing of the products by ^{19}F NMR

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Abstract

2-Cyclopentyl- and 2-cyclohexyl-6-fluorobenzoxazole were prepared and microbially hydroxylated employing *Cunninghamella blakesleeana* DSM 1906 and *Bacillus megaterium* CCM 2037. The conversions mimic those previously observed with benzoxazoles not containing fluorine atoms. The fluorinated substrates have the advantage that the course of hydroxylation and in addition any side reactions, can be investigated by ^{19}F NMR spectroscopy. ^{19}F NMR spectra could be measured directly from the fermentation samples which had neither been extracted nor chromatographed, consequently minimising the loss of any material.

Keywords: NMR spectroscopy; Microbial hydroxylation; Benzoxazoles; *Cunninghamella blakesleeana*; *Bacillus megaterium*

1. Introduction

1.1. Earlier work

We have recently shown that benzoxazoles derived from alicyclic carboxylic acids are good substrates for the hydroxylating enzymes of *Cunninghamella blakesleeana* DSM 1906 and *Bacillus megaterium* CCM 2037 [1–4]. The benzoxazole moiety serves as a protecting group for the carboxylic acid which can be readily removed after successful hydroxylation. A common approach to obtain the desired hydroxylated products from a given substrate is to screen a large range of bacteria and fungi until the best suited is found. Our goal, however, was different since we wanted to obtain products from a range of substrates employing only a limited number of microorganisms. An additional limitation of bioconversions is that the conditions employed by default might not be those which give the optimal result in terms of product yield or optical purity. We found, for example, that the fermentation products we obtained differed with respect to yields and e.e. when changing conditions such as fermentation time, dissolved oxygen tension and pH-value [5–7]. One major problem concerning microbial hydroxylations is the low yield of product often found with these bioconversions, especially with longer fermentation times. An explanation for these observa-

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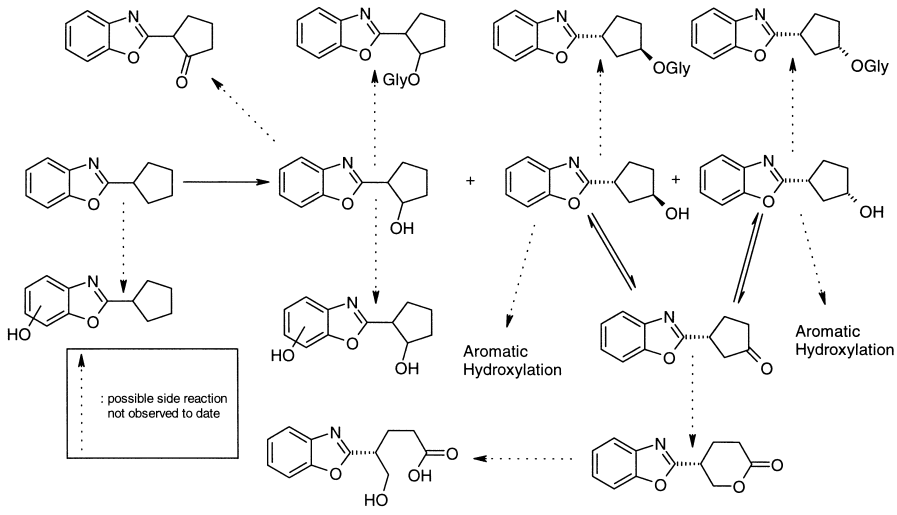


Scheme 1. Bioconversion of cyclopentylbenzoxazole using *C. blakesleeana* DSM 1906; potential side reactions are indicated with arrows; Gly indicates glycosylated compounds.

tions is that follow-up reactions are going on in the cells which further transform initially produced compounds. The oxidation of the main alcohol formed from 2-cyclopentylbenzoxazole as shown in Scheme 1 is a good example for this. Possible follow-up reactions we speculated about were Baeyer–Villiger oxidations of the ketone, glycosylation of alcohols or hydroxylation of the aromatic portion of the benzoxazole [8–12]. Furthermore, all these reactions would furnish compounds that were highly polar and water soluble and therefore would escape the normal work-up procedure. We reasoned that if we could find a method to detect these reactions we also might be able to control them and thereby increase the yield of desired product.

1.2. Use of fluorobenzoxazoles

In order to be able to investigate if these or other reactions take place we considered employing benzoxazoles containing a fluorine atom in the aromatic part of the molecule. This would allow the measurement of ^{19}F NMR spectra of isolated compounds or of product mixtures. ^{19}F NMR sensitivity is comparable to ^{1}H NMR spectra due to the magnetic moment and 100% abundance of ^{19}F. However, an additional advantage is that all the fluorine found only derives from the substrate and therefore acts as a marker and allows the tracing of the benzoxazole components during the fermentation. In addition, there exists the possibility to obtain structural information about the product by examining the coupling pattern of the ^{19}F-signal in coupled spectra due to neighbouring protons. In cases where aromatic hydroxylation would occur, there would also be a change of this pattern due to loss of one proton in the vicinity of the ^{19}F. Furthermore, solvent suppression does not have to be used in eliminating the dynamic range problem encountered in the proton spectra of aqueous solutions. ^{19}F-signals are spread over a large range of frequencies making the overlapping of lines less likely than compared with proton spectra [13].

1.3. Issues to be addressed

(A) Would the benzoxazoles containing a fluorine atom be converted in the same way as the normal benzoxazoles? Is this system therefore a true model?

(B) Provided that fluorinated benzoxazoles are converted satisfactorily, would the ^{19}F NMR give the desired information? The fluorine atom

observed is quite far apart from the atoms that are converted within the molecule. Would it be possible to distinguish the signals of the products obtained in ^{19}F NMR?

2. Results and discussion

2.1. Preparation of starting materials and microbial hydroxylation

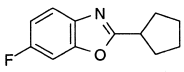
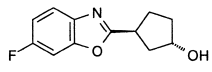
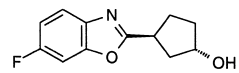
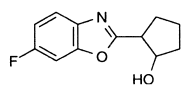
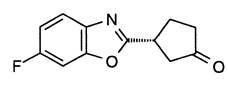
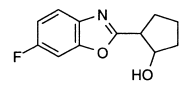
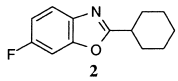
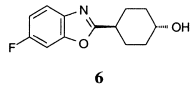
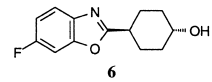
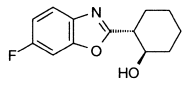
The synthetically most interesting compounds, 2-cyclopentyl- and 2-cyclohexyl-6-fluorobenzoxazoles **1** and **2** were chosen as substrates because they had been intensively studied as non-fluorinated analogues. Commercially available 5-fluoro-2-nitrophenol was hydrogenated at ambient temperature using palladium on carbon (5%) to give the corresponding aminophenol [14]. This was then reacted with the respective carboxylic acid in an analogous manner to the non-fluorinated benzoxazoles [2]. Substrates **1** and **2** were hydroxylated using *C. blakesleeana* DSM 1906 and *B. megaterium* CCM 2037. The products obtained are shown in Table 1.

As can be seen, the products formed are analogous to those formed with benzoxazoles not carrying a fluorine atom. A closer examination of the table shows that yield and enantiomeric excess of these compounds are also comparable with previous findings. This suggests that the fluorobenzoxazoles are converted by the same mechanisms as the nonsubstituted compounds, making them true models of that system. Encouraged by this observation, we undertook ^{19}F NMR measurements.

2.2. ^{19}F NMR investigations

The ^{19}F chemical shifts of substrates **1** and **2**, measured in $CDCl_3$ relative to CCl_3F, were -116.89 ppm and -116.87 ppm, respectively. Alcohols **3** (-116.55), **4** (-116.67), **6**

Table 1
Products formed and isolated by microbial hydroxylations of substrates **1** and **2**

Substrate	Products with *Bacillus megaterium* CCM 2037		Products with *Cunninghamella blakesleeana* DSM 1906	
 **1**	 **3**	yield: 55 % e.e.: 31 %	 **3**	yield: 36 % e.e.: 51 %
	 **4**	yield:18 %	 **5**	yield: 4 % e.e.: 58 %
			 **4**	yield: 0.2 % e.e.: 40 %
 **2**	 **6**	yield: 34 %	 **6**	yield: 32 %
	 **7**	yield: 12 %		

Absolute configurations have been assigned by comparison of magnitude and sign of optical rotation with earlier work [2]. Enantiomeric excess was measured by HPLC using a chiral column as described in [3]. A small amount of *cis*-**3** was also formed and could be detected by HPLC or ^{19}F NMR. Isolated yields are given.

(−116.49) and **7** (−116.18) as well as ketone **5** (−115.70) displayed different values. The signals ranged from −115.7 to −116.9 ppm or roughly 340 Hz on a 300 MHz NMR spectrometer. Therefore it should be possible to distinguish between them in mixtures, provided that the resolution is good enough. This finding is quite surprising since the fluorine atom is in the aromatic part of the molecule and the structural differences in compounds **1–7** are only in the cycloalkyl portion.

The next step was to look at actual fermentation extracts: Fig. 1 shows a spectrum of a concentrated ethyl acetate extract from a fermentation of **2** with *B. megaterium* after the residue was redissolved in $CDCl_3$. In this case all the products can be distinguished. The coupled spectrum shows that the benzoxazole part of all the products is still intact. The substrate **2** and the main products **6** and **7** can be recognised. However,two compounds not previously observed are also formed in small amounts.

Fig. 2 shows a very interesting result. In the fermentation of **1** with *B. megaterium* formation of the main product **3** and the ketone **5** can be seen. However, an additional product can be found with a signal at −122.8 ppm. Expansion of the coupled spectrum (see Fig. 2B) shows that this signal has a different coupling pattern compared with the fluorobenzoxazoles. This suggests that one proton at the aromatic ring has been substituted, presumably by a hydroxyl group.

Despite our initial fears that the aqueous solutions might be too dilute and would, therefore, not allow the observation of ^{19}F NMR-signals, we were pleased to find that even in these cases ^{19}F NMR spectra could be measured albeit over a longer time period. Fig. 3A shows a decoupled spectrum of a fermentation broth simply decanted from cells and a capillary of D_2O added in order to obtain a lock signal. The total time of acquisition was 2 h and a clean spectrum was obtained. The measurement of the decoupled spectrum of the same sample took an overnight run and also gave clean signals.

Interestingly, this spectrum also shows a fluorine signal with a coupling pattern different from the benzoxazoles. The singlet at −119.5 ppm is fluoride ion, released from the aromatic part of the benzoxazole [15,16]. This would also suggest a pathway of aromatic hydroxylation

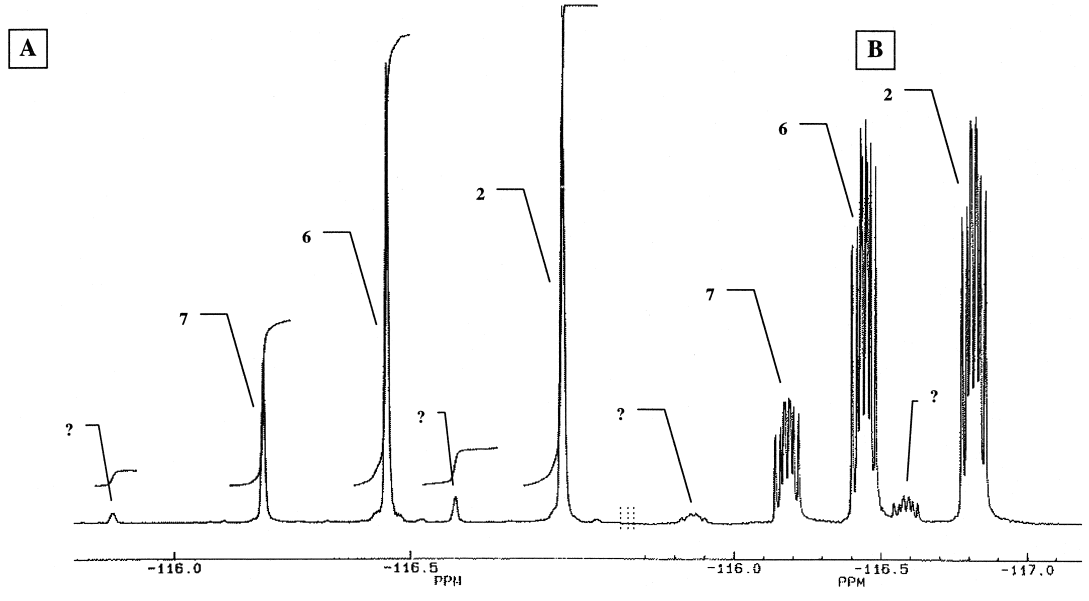


Fig. 1. A and B: ^{19}F NMR spectrum of a fermentation extract of **2** with *B. megaterium* dissolved in $CDCl_3$. A is the proton decoupled, B the coupled spectrum. 16 scans were accumulated.

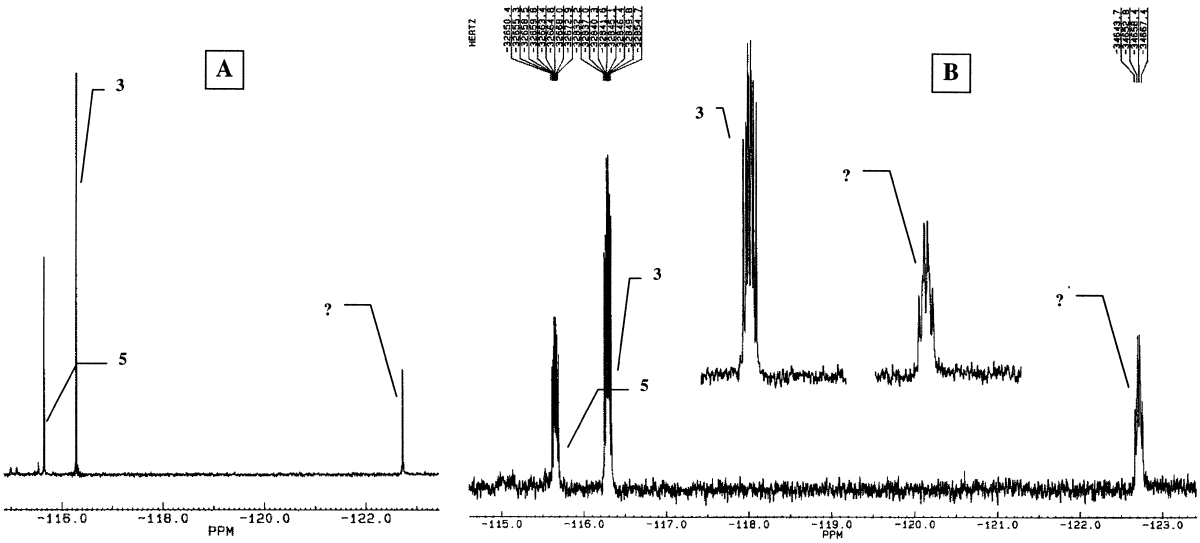


Fig. 2. A and B: ^{19}F NMR spectrum of a fermentation extract of **1** with *B. megaterium* dissolved in $CDCl_3$. A is the proton decoupled, B the coupled spectrum. 16 Scans were accumulated. The ratio of alcohol **3** and ketone **5** does not correspond with yields in Table 1 because this sample was prepared from a column fraction after flash chromatography.

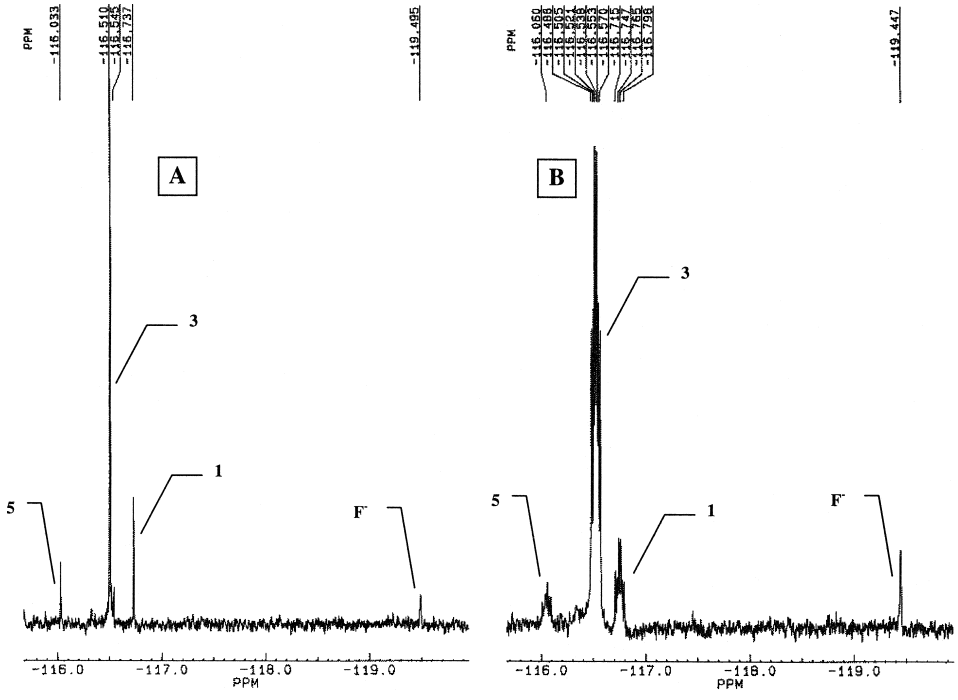


Fig. 3. A and B: ^{19}F NMR spectrum of the broth from the fermentation of **1** with *C. blakesleena* 17 h after substrate addition. A capillary of D_2O was added for locking. 2048 scans were accumulated for A, 10240 scans for B.

followed by elimination of fluoride ion. Subsequently, experiments were undertaken that confirmed that fluoride ion was not present in the medium and that the amount of fluoride ion increased with a longer fermentation time.

3. Summary and outlook

We have shown that ^{19}F NMR spectra of the fermentation products of fluorinated benzoxazoles can be measured quite easily and that they give additional information that cannot be gathered using conventional chromatographic analysis techniques. We believe that these measurements will be very useful in order to find out which additional reactions are catalysed by the enzyme systems of the microorganisms under consideration. Furthermore, by measuring at different time intervals during the fermentations, we should be able to find ways to control the course of the hydroxylation with respect to the formation or destruction of products.

4. Experimental

4.1. ^{19}F NMR measurements

Measurements were carried out on a BRUKER MSL 300 at 282.400 MHz. Both coupled and proton decoupled spectra were measured. In the case of isolated compounds or fermentation extracts $CDCl_3$ was used as solvent. In the case of fermentation samples, the aqueous solution itself was used and a capillary of D_2O was introduced into the sample tube or 10% D_2O was added.

4.2. Analytical methods

Melting points (uncorrected): Büchi 530. *Optical rotation*: DIP-370 Digital Polarimeter (Japan Spectroscopic) ^1H, ^{13}C NMR: Gemini 200 (Varian), MSL 300 (Bruker) solvent as internal standard. *Elemental analyses*: Microanalytical Laboratory of the Institute for Inorganic Chemistry, TU Graz. *HPLC for determination of e.e.*: JASCO system containing pump 880-PU, UV-detector 875-UV and AXXIOM Model 727 chromatography software; chiral column: CHIRALPAK AD from DAICEL with the eluent heptane/2-propanol 95:5. Detection of fluorobenzoxazoles was conducted at 230 nm as described in Ref. [3].

4.3. General procedure for 6-fluorobenzoxazole preparation [2]

A solution of 2-amino-5-fluorophenol (10 mmol), PPE (10 g) and carboxylic acid (10 mmol) in dichloromethane (50 ml) is refluxed overnight. Then the reaction mixture is cooled with ice, water (100 ml) is added and the pH is adjusted to 7 by carefully adding solid $NaHCO_3$. Separation of the phases and extraction of the water phase with dichloromethane (3×100 ml) gives a combined organic layer, which is dried (Na_2SO_4) and evaporated. Further purification of the crude product is carried out by column chromatography on silica gel (eluent: cylohexane/ethyl acetate 3:1) and kugelrohr distillation.

*4.3.1. 2-Cyclopentyl-6-fluorobenz-1,3-oxazole (**1**)*

Yield: 52%; oil, ^1H NMR ($CDCl_3$): ppm 1.6–2.3 (m, 8H, H-2, H-3, H-4, H-5), 3.35 (p, $J = 7.9$ Hz, 1H, H-1), 7.01 (dt, $J = 9.0$ Hz, $J = 2.4$ Hz, 1H, H-5′), 7.17 (dd, $J = 8.1$ Hz, $J = 2.4$ Hz, 1H, H-7′), 7.56 (dd, $J = 8.6$ Hz, $J = 4.9$ Hz, 1H, H-4′). ^{13}C NMR ($CDCl_3$): ppm 25.68 (C-3, C-4), 31.33 (C-2, C-5), 33.38 (C-1), 98.87 (d, $^2J_{C,F} = 27.9$ Hz, C-7′), 111.78 (d, $^2J_{C,F} = 24.4$ Hz, C-5′), 119.66 (d, $^3J_{C,F} = 10.5$ Hz, C-4′), 137.61(d, $^4J_{C,F} = 1.7$ Hz, C-9′), 150.77 (d, $^3J_{C,F} = 14.7$ Hz, C-8′), 160.25 (d, $^1J_{C,F} = 241.4$ Hz, C-6′), 171.26 (C-2′).^{19}F NMR ($CDCl_3$): ppm -116.89. Anal. calcd. for $C_{12}H_{12}FNO$, C: 70.2 H: 5.89; found: 70.3 H: 5.91.

*4.3.2. 2-Cyclohexyl-6-fluorobenz-1,3-oxazole (**2**)*

Yield: 58%; mp 61–62°C; ^{1}H NMR ($CDCl_3$): ppm 1.30–2.20 (m, 10H, H-2, H-3, H-4, H-5, H-6), 2.95 (tt, $J = 11.1$ Hz, $J = 3.6$ Hz, 1H, H-1), 7.05 (dt, $J = 9.2$ Hz, $J = 2.3$ Hz, 1H, H-5′), 7.2 (dd, $J = 8.0$ Hz, $J = 2.3$ Hz, 1H, H-7′), 7.6 (dd, $J = 8.6$ Hz, $J = 4.9$ Hz, 1H, H-4′). ^{13}C NMR ($CDCl_3$): ppm 25.59 (C-3, C-5), 25.77 (C-4), 30.43 (C-2, C-6), 37.91 (C-1), 98.41 (d, $^{2}J_{C,F} = 28$ Hz, C-7′), 111.81 (d, $^{2}J_{C,F} = 25$ Hz, C-5′), 119.78 (d, $^{3}J_{C,F} = 10$ Hz, C-4′), 137.57 (d, $^{4}J_{C,F} = 1.5$ Hz, C-9′), 150.54 (d, $^{3}J_{C,F} = 14.0$ Hz, C-8′), 160.28 (d, $^{1}J_{C,F} = 241$ Hz, C-6′) 171.24 (C-2′). ^{19}F NMR ($CDCl_3$): ppm −116.87. Anal. calcd. for $C_{13}H_{14}FNO$, C: 71.2 H: 6.44; found: 71.1 H: 6.48.

4.4. General procedure for the fermentations

Transformations with *C. blakesleeana* were performed in 1-l shaking flasks containing 250 ml medium E. After 2 days of growth, an ethanolic solution of substrate (0.3 g/l) was added to the culture.

Biohydroxylations with *B. megaterium* were carried out in 1-l shaking flasks containing 250 ml of medium K. After 2–4 days the culture broth was extracted twice with ethyl acetate. The organic phase was evaporated after drying with Na_2SO_4. Products were separated by column chromatography on silica gel (eluent: cyclohexane/ethyl acetate 3:1).

Medium E consisted (per liter) of 5 g of malt extract (Merck), 10 g of glucose, 2 g of peptone (Merck), and 2 g of yeast extract (Oxoid). Medium K was made of 4.5 g/l $Na_2HPO_4 \cdot 2H_2O$, 1.5 g/l KH_2PO_4, 3. g/l $(NH_4)_2SO_4$, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.05 g/l Fe(III)–NH_4-citrate, 0.02 g/l $CaCl_2 \cdot 2H_2O$, 1 g/l Na-acetate, 1 g/l yeast extract, 20 g/l glucose, 1 ml/l trace elements solution.

*4.4.1. (1S,3S)-3-(6-Fluoro-1,3-benzoxazol-2-yl)cyclopentan-1-ol (**3**)*

Yield: 36% (C. b.), 59% (B. m), mp 46–48°C, $[\alpha]_D^{20} + 14.6$ (c 0.5, dichloromethane) e.e. 51%; ^{1}H NMR ($CDCl_3$): ppm 1.70–2.45 (m, 6H, H-2, H-4, H-5), 3.70 (p, $J = 8$ Hz, 1H, H-1), 4.58 (m, 1H, H-3), 7.05 (dt, $J = 9$ Hz, $J = 2.4$ Hz, 1H, H-5′), 7.20 (dd, $J = 8.1$ Hz, $J = 2.4$ Hz, 1H, H-7′), 7.55 (dd, $J = 8.8$ Hz, $J = 4.9$ Hz, 1H, H-4′). ^{13}C NMR ($CDCl_3$): ppm 29.09 (C-4), 35.21 (C-5), 36.83 (C-2), 40.93 (C-1), 73.46 (C-3), 98.63 (d, $^{2}J_{C,J}$, = 28.7 Hz, C-7′), 112.13 (d, $^{2}J_{C,F} = 25.7$ Hz, C-5′), 119.89 (d, $^{3}J_{C,F} = 9.8$ Hz, C-4′), 137.70 (C-9′), 151.01 (d, $^{3}J_{C,F} = 13.4$ Hz, C-8′), 160.54 (d, $^{1}J_{C,F} = 243.8$ Hz, C-6′), 170.99 (C-2′). ^{19}F NMR ($CDCl_3$): ppm −116.55. Anal. calcd. for $C_{12}H_{12}FNO_2$, C: 65.2 H: 5.47; found: 65.3 H: 5.51.

*4.4.2. cis-3-(6-Fluoro-1,3-benzoxazol-2-yl)cyclopentan-1-ol (**3a**)*

^{13}C NMR ($CDCl_3$): ppm 29.93 (C-4), 35.85 (C-5), 37.20 (C-2), 40.30 (C-1), 73.49 (C-3), 98.63 (d, $^{2}J_{C,F} = 28.7$ Hz, C-7′), 112.13 (d, $^{2}J_{C,F} = 25.7$ Hz, C-5′), 119.89 (d, $^{3}J_{C,F} = 9.8$ Hz, C-4′), 137.70 (C-9′), 151.01 (d, $^{3}J_{C,F} = 13.4$ Hz, C-8′), 160.54 (d, $^{1}J_{C,F} = 243.8$ Hz, C-6′), 171.43 (C-2′). ^{19}F NMR ($CDCl_3$): ppm −116.30.

*4.4.3. 2-(6-Fluoro-1,3-benzoxazol-2-yl)cyclopentan-1-ol (**4**)*

Yield: 31% (B. m.), 0.2% (C. b.), oil, ^{1}H NMR ($CDCl_3$): ppm 1.60–2.20 (m, 6H), 3.28 (q, 1H), 4.60 (q, 1H), 7.10 (dt, 1H, H-5′), 7.22–7.40 (m, 1H, H-7′), 7.60–7.67 (dd, 1H, H-4′). ^{19}F NMR ($CDCl_3$): ppm −116.67.

*4.4.4. (R)-3-(6-fluoro-1,3-benzoxazol-2-yl)cyclopentan-1-on (**5**)*

Mp 99–103°C; $[\alpha]_D^{20}$ −14.5 (c 0.5, dichloromethane) e.e. 58%; ^{1}H NMR ($CDCl_3$): ppm 2.30–2.65 (m, 4H, H-4, H-5), 2.75 (d, 2H, H-2), 3.77 (m, 1H, H-1), 7.10 (dt, $J = 9$ Hz, $J = 2.4$ Hz, 1H, H-5′), 7.25 (dd, $J = 7.9$ Hz, $J = 2.3$ Hz, 1H, H-7′), 7.60 (dd, $J = 8.8$ Hz, $J = 4.9$ Hz, 1H, H-4′). ^{13}C NMR ($CDCl_3$): ppm 28.06 (C-5), 36.11 (C-4), 37.73 (C-2), 42.58 (C-1), 98.86 (d, $^{2}J_{C,F} = 28.7$ Hz, C-7′), 112.62 (d, $^{2}J_{C,F} = 25.7$ Hz, C-5′), 120.40 (d, $^{3}J_{C,F} = 9.8$

Hz, C-4′), 137.60 (C-9′), 151.11 (d, $^{3}J_{C,F} = 15.1$ Hz, C-8′), 160.84 (d, $^{1}J_{C,F} = 243.9$ Hz, C-6′), 168.29 (C-2′), 215.84 (C-3). ^{19}F NMR ($CDCl_3$): ppm −115.70. Anal. calcd. for $C_{12}H_{10}FNO_2$, C: 65.8 H: 4.60; found: 65.9 H: 4.63.

*4.4.5. trans-4-(6-Fluoro-1,3-benzoxazol-2-yl)cyclohexan-1-ol (**6**)*

Mp 138–140°C; ^{1}H NMR ($CDCl_3$): ppm 1.45–2.35 (m, 8H, H-2, H-3, H-5, H-6), 2.5–2.6 (sb, 1H, OH), 2.87 (tt, $J = 11.7$ Hz, $J = 3.7$ Hz, 1H, H-1), 3.71 (tt, $J = 10.4$ Hz, $J = 4.2$ Hz, 1H, H-4), 7.03 (dt, $J = 7$ Hz, $J = 2.4$ Hz, 1H, H-5′), 7.15 (dd, $J = 8.0$ Hz, $J = 2.4$ Hz, 1H, H-7′), 7.55 (dd, $J = 8.8$ Hz, $J = 4.9$ Hz, 1H, H-4′). ^{13}C NMR ($CDCl_3$): ppm 28.54 (C-2, C-6), 34.59 (C-3, C-5), 36.95 (C-1), 69.58 (C-4), 98.46 (d, $^{2}J_{C,F} = 28$ Hz, C-7′), 111.98 (d, $^{2}J_{C,F} = 24$ Hz, C-5′), 119.79 (d, $^{3}J_{C,F} = 10$ Hz, C-4′), 137.28 (C-9′), 160.29 (d, $^{1}J_{C,F} = 239$ Hz, C-6′) 171.12 (C-2′). ^{19}F NMR ($CDCl_3$): ppm −116.49. Anal. calcd. for $C_{13}H_{14}FNO_2$, C: 66.4 H: 6.00; found: 66.5 H: 5.99.

*4.4.6. (1R,2R)-2-(6-Fluoro-1,3-benzoxazol-2-yl)cyclohexan-1-ol (**7**)*

Yield: 12%, oil, ^{1}H NMR ($CDCl_3$) ppm 1.39–1.58 (m, 4H, H-5, H-6) 1.82 (m, 2H, H-4), 2.08–2.35 (m, 2H, H-3), 2.85 (m, 1H, H-1), 3.15 (sb, 1H, OH), 3.97 (m, 1H, H-2), 7.05 (dt, $J = 7$ Hz, $J = 2.4$ Hz, 1H, H-5′), 7.20 (dd, $J = 8.0$ Hz, $J = 2.4$ Hz, 1H, H-7′), 7.58 (dd, $J = 8.8$ Hz, $J = 4.9$ Hz, 1H, H-4′). ^{13}C NMR ($CDCl_3$): ppm 24.43 (C-6), 25.10 (C-5), 29.15 (C-4), 33.83 (C-3), 46.14 (C-1), 71.65 (C-2), 98.42 (d, $^{2}J_{C,F} = 28$ Hz, C-7′), 111.91 (d, $^{2}J_{C,F} = 24$ Hz, C-5′), 119.72 (d, $^{3}J_{C,F} = 10$ Hz, C-4′), 137.33 (C-9′), 160.31 (d, $^{1}J_{C,F} = 239$ Hz, C-6′), 171.24 (C-2′). ^{19}F NMR (CDCl3): ppm −116.18. Anal. calcd. for $C_{13}H_{14}FNO_2$, C: 66.4 H: 6.00; found: 66.3 H: 6.03.

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