R and 0.10 mL of 5.8 mM NADPH in a cuvette in the sample chamber of a Cary 118 UV–vis spectrometer set at 340 nm at room temperature. Then, an aliquot of inhibitor was added, followed by 10 μL of DHFR. The initial steady rate of A(340) decrease was measured.

Calculation of K_i. The following equation was used: $K_i(\text{app}) = i_{50}[1/(1+(s/K_s))]$, where s was the concentration of H_2-folate in M in cuvette, K_s was the Michaelis constant for H_2-folate (440 nM), and $i_{50} = i[1/([v(i = o)/v(i = i)]) - 1)]$ where i is the concentration of inhibitor in nM, i_{50} is the concentration of inhibitor that gave 50% inhibition of DHFR activity, $v(i = o)$ is the rate of decrease of A(340) without inhibitor, and $v(i = i)$ is the rate of decrease of A(340) in the presence of inhibitors at concentration i. DHFR assays and calculations for each inhibitor were repeated three to seven times until the standard error in K_i was less than 20%.

QSAR Parameters. All parameters of Table I come from the compilation used in ref 9 unless noted.

Acknowledgment. This project was supported by the Science Fund of the Chinese Academy of Sciences. Compounds **45–58** of Table I were synthesized by Fang Zhao-Xia of Department of Medicinal Chemistry, Beijing Medical University.

Registry No. 1, 80407-57-4; **2**, 80407-60-9; **3**, 80407-61-0; **4**, 7319-45-1; **5**, 69945-52-4; **6**, 69945-57-9; **7**, 77113-60-1; **8**, 77113-56-5; **9**, 69945-50-2; **10**, 77113-54-3; 11, 836-06-6; **12**, 80407-62-1; **13**, 80407-59-6; 14, 18588-43-7; **15**, 71525-05-8; **16**, 77113-55-4; **17**, 46726-70-9; 18, 80416-29-1; **19**, 77113-61-2; **20**, 80407-58-5; **21**, 49561-94-6; **22**, 77113-57-6; **23**, 69945-58-0; **24**, 69945-56-8; **25**, 69945-51-3; **26**, 69945-55-7; **27**, 20285-70-5; **28**, 77113-63-4; **29**, 77113-62-3; **30**, 77113-59-8; **31**, 69945-53-5; **32**, 77113-58-7; **33**, 59481-28-6; **34**, 69945-59-1; **35**, 69945-54-6; **36**, 69945-60-4; **37**, 50823-94-4; **38**, 73356-41-9; **39**, 30077-60-2; **40**, 50823-96-6; **41**, 5355-16-8; **42**, 53808-87-0; **43**, 20344-69-8; **44**, 738-70-5; **45**, 78233-99-5; **46**, 107697-99-4; **47**, 73356-40-8; **48**, 83158-06-9; **49**, 107698-00-0; **50**, 7334-22-7; **51**, 111743-19-2; **52**, 107698-01-1; **53**, 49873-11-2; **54**, 13932-40-6; **55**, 100515-03-5; **56**, 107697-98-3; **57**, 80267-19-2; 58, 111743-20-5; **59**, 100515-04-6; **60**, 107697-96-1; **61**, 107697-97-2; DHFR, 9002-03-3.

N-Aryl 3-Halogenated Azetidin-2-ones and Benzocarbacephems, Inhibitors of β-Lactamases

Roger Joyeau,† Huguette Molines,† Roger Labia,‡ and Michel Wakselman*†

CNRS-CERCOA, 2-8, rue Henri Dunant, 94320 Thiais, France, and Museum National d'Histoire Naturelle, 63, rue Buffon, 75005 Paris, France. *Received May 18, 1987*

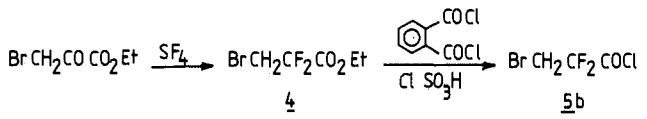
N-(3-Carboxy-6-methylphenyl)-3-fluoroazetidin-2-one and a series of related *N*-aryl-3-halo- and -3,3-dihaloazetidinones **3**, in which the halo substituent is a fluorine or a bromine atom, were prepared, by using the Wasserman procedure of cyclization of β-bromopropionamides as a key step. Their affinities for the TEM-1 β-lactamase were determined and compared with those of a series of tricyclic azetidinones, the benzocarbacephems **2**, and known β-lactamase inhibitors. The β-lactams **2** and **3** behave as competitive inhibitors and not as substrates of the enzyme; neither halogen substitution (series **3**) nor ring strain (series **2**) induces enzymatic hydrolysis.

In spite of the introduction in therapy of innovative β-lactams with expanded antibacterial activity and improved β-lactamase stability, bacteria continue to exhibit resistance. Therefore enzymatic inactivation of the newer molecules is still a major problem. A recent outbreak of bacteria carrying plasmid-mediated β-lactamases markedly active against third-generation cephalosporins has renewed the interest in β-lactamase inhibitors.[1] Clavulanic acid, sulbactam, and related compounds[2] are β-lactamase inhibitors which potentiate the antibacterial activity of various β-lactams, but the need for β-lactamase inhibitors of new chemical structures and new biological properties remains a subject of very high interest.

We have previously shown that some *N*-arylazetidinones, and particularly the 3-carboxy-6-methyl-substituted one, **1a** (Figure 1), are interesting competitive inhibitors of β-lactamases. However, they are not detectable substrates of the enzymes.[3] Their bromomethylated analogues such as **1b** are not suicide inhibitors,[3] for ring opening is a prerequisite for the design of new suicide inhibitors of β-lactamases.[2]

To increase the reactivity of the β-lactam ring toward enzymatic ring opening, two ways were considered: cyclization to give strained tricyclic β-lactams **2** and halogen substitution α to the carbonyl to give compounds of type **3** (vide infra). We have already described the synthesis

Scheme I



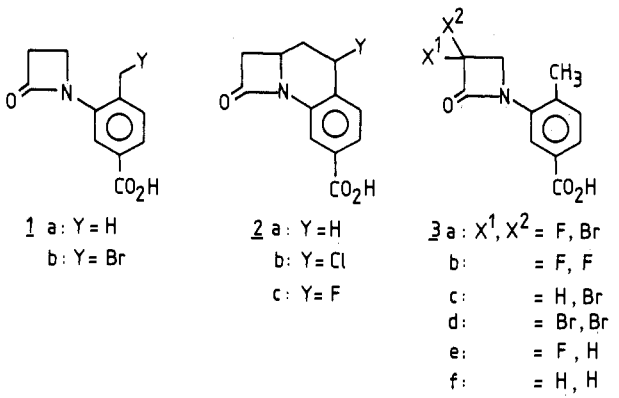
of the benzocarbacephems **2** (Y = H, Cl, or F).[4] We report here the results of their biological activity together with the synthesis and properties of the *N*-aryl 3-halogenated and 3,3-dihalogenated azetidinones **3** (X^1, X^2 = H, Br, or F).

The presence of one or two halogen substituents α to the carbonyl should increase the $\nu_{C=O}$ IR stretching frequency, one of the criteria of the reactivity of the β-lactam ring.[5] In this respect, fluorine substitution, which will not

† CNRS-CERCOA.
‡ Museum National d'Histoire Naturelle.

(1) Sirot, D.; Sirot, J.; Labia, R.; Morand, A.; Courvalin, P.; Darfeuille-Michaud, A.; Perroux, R.; Cluzel, R. *J. Antimicrob. Chemother.* **1987**, *20*, 323.
(2) (a) Knowles, J. R. *Acc. Chem. Res.* **1985**, *18*, 97. (b) Chen, Y. L.; Chang, C. W.; Hedberg, K.; Guarino, K.; Welch, W. M.; Kiessling, L.; Retsema, J. A.; Haskell, S. L.; Anderson, M.; Manousos, M.; Barrett, J. F. *J. Antibiot.* **1987**, *40*, 803. (c) Micetich, R. G.; Maiti, S. N.; Spevak, P.; Hall, T. W.; Yamabe, S.; Ishida, N.; Tanaka, M.; Yamazaki, T.; Nakai, A.; Ogawa, K. *J. Med. Chem.* **1987**, *30*, 1469.
(3) Zrihen, M.; Labia, R.; Wakselman, M. *Eur. J. Med. Chem.—Chim. Ther.* **1983**, *18*, 307.
(4) (a) Joyeau, R.; Dugenet, Y.; Wakselman, M. *J. Chem. Soc., Chem. Commun.* **1983**, 431. (b) Joyeau, R.; Yadav, L. D. S.; Wakselman, M. *J. Chem. Soc., Perkin Trans. 1* **1987**, 1899.

0022-2623/88/1831-0370$01.50/0



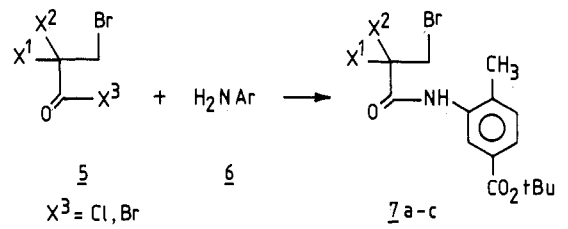
1 a: Y = H
b: Y = Br

2 a: Y = H
b: Y = Cl
c: Y = F

3 a: X¹, X² = F, Br
b: = F, F
c: = H, Br
d: = Br, Br
e: = F, H
f: = H, H

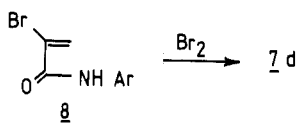
Figure 1.

Scheme II



5 (X³ = Cl, Br) + 6 → 7 a-c

Scheme III



8 → 7 d

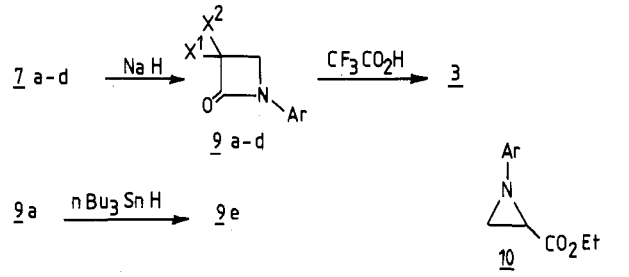
introduce a large steric hindrance, is particularly interesting for a possible biological effect. Only a few examples of 3-fluorinated azetidinones are known.[6]

Chemistry

Cyclization of β-halopropionamides is an efficient method for the synthesis of β-lactams.[7,8] Use of NaH as a base in a diluted CH_2Cl_2–DMF solution reduces the amount of the acrylamide side product formed by a competitive β-elimination reaction.[8] This procedure has been now applied for the preparation of *N*-aryl-3-halo- and -3,3-dihaloazetidin-2-ones **3**.

Most of the starting sustituted propionyl halides **5** are easily available. 2,3-Dibromo-2-fluoropropanoyl bromide (**5a**) was prepared by following ref 9. 3-Bromo-2,2-difluoropropanoyl chloride (**5b**) was unknown and was obtained from ethyl 3-bromopyruvate by treatment with sulfur tetrafluoride[10] and chlorination of the obtained ethyl 3-bromo-2,2-difluoropropanoate (**1**) by Middleton's exchange procedure[12] (Scheme I).

Scheme IV



7 a-d → (NaH) 9 a-d → (CF₃CO₂H) 3

9a → (nBu₃SnH) 9e

10

Condensation of these acid halides with *tert*-butyl 3-amino-4-methylbenzoate (**6**) in the presence of 2,6-dimethylpyridine gave the substituted 3-bromopropionanilides **7a–c** (Scheme II).

In the case of the 2,3-dibromopropanoyl chloride, the yield was lower because a β-elimination also led to the acrylanilide **8** as a side product. This compound was useful for the preparation of the substituted 2,2,3-tribromopropionanilide **7d** by addition of bromine (Scheme III).

Cyclization of the 3-bromo-2,2-dihalopropionanilides **7** occurred easily and gave the azetidinones **9a–d** (Scheme IV). As expected, the yield was lower in the case of the 2,3-dibromopropionanilide **7c**, which has an acidic α-hydrogen atom. In this reaction, dichloromethane free of ethanol is required,[8] as traces of this alcohol during the cyclization of **7c** lead to the formation of the aziridine **10** as a side product.[13]

Reductive debromination of the 3-bromo-3-fluoroazetidinone **9a** with tri-*n*-butyltin hydride[14] quantitatively afforded the 3-fluoroazetidinone **9e**.

The cleavage of the *tert*-butyl protecting group of **9a–e** with trifluoroacetic acid provided the corresponding free acids **3a–e**. As no asymmetric induction step is included in the synthetic scheme, the acids unsymmetrically substituted at the 3-position were obtained as racemic mixtures. The observed high $\nu_{C=O}$ IR frequencies (1765–1790 cm^{-1}) of azetidinones **3a–e** indicate a rather high potential reactivity of the β-lactam carbonyl.[5]

Biology

The synthetic β-lactams, clavulanic acid, sulbactam (suicide inhibitors), and cloxacillin (reversible inhibitor) were tested as substrates and inhibitors for a partially purified preparation of the plasmid-mediated broad-spectrum TEM-1 β-lactamase, coded by the plasmid pBR 322. We used a computerized microacidimetric β-lactamase assay[15] which yields to K_m and V_{max} for substrates. A modified procedure was used for inhibitors (see Experimental Section). The nine new compounds belonging to the series **2** and **3** were not substrates for the TEM-1 β-lactamase, when tested at the concentration of 50 μg/mL (susceptibility limit 0.3% of penicillin G hydrolysis). A similar observation was made for clavulanic acid and

(5) (a) Morin, R. B.; Jackson, B. G.; Mueller, R. A.; Lavagnino, E. R.; Scanlon, W. B.; Andrews, S. L. *J. Am. Chem. Soc.* **1969**, *91*, 1401. (b) Indelicato, J. M.; Norvilas, T. P.; Pfeiffer, R. R.; Wheeler, W. J.; Wilham, W. L. *J. Med. Chem.* **1974**, *17*, 523. (c) Butler, A. R.; Freeman, A. K.; Wright, D. E. *J. Chem. Soc., Perkin Trans. 2* **1977**, 765. (d) Coene, B.; Schanck, A.; Dereppe, J. M.; Van Meerssche, M. *J. Med. Chem.* **1984**, *27*, 694. (e) Page, M. I. *Acc. Chem. Res.* **1984**, *17*, 144. (f) Costerousse, G.; Teutsch, G. *Tetrahedron* **1986**, *42*, 2685. (g) Boyd, D. B.; Eigenbrot, C.; Indelicato, J. M.; Miller, M. J.; Pasini, C. E.; Woulfe, S. R. *J. Med. Chem.* **1987**, *30*, 528.

(6) (a) Tada, K.; Toda, F. *Tetrahedron Lett.* **1978**, 563. (b) Kellog, M. S.; Hamanaka, E. S. Ger. Offen. 3,00316; *Chem. Abstr.* **1981**, *94*, 84113*r*. (c) Doherty, J. B., et al. *Nature (London)* **1986**, *322*, 192.

(7) Sebti, S.; Foucaud, A. *Synthesis* **1983**, 546 and references therein.

(8) (a) Wasserman, H. M.; Hlasta, D. J.; Temper, A. W.; Wu, J. S. *Tetrahedron Lett.* **1979**, 549. (b) Wasserman, H. M.; Hlasta, D. J.; Temper, A. W.; Wu, J. S. *J. Org. Chem.* **1981**, *46*, 2999.

(9) Molines, H.; Nguyen, T.; Wakselman, C. *Synthesis* **1985**, 755.

(10) Treatment with DAST[11] afforded a mixture of several fluorinated products.

(11) (a) Middleton, W. J.; Bingham, E. M. *J. Org. Chem.* **1980**, *45*, 2883. (b) Kobayashi, Y.; Sai, H.; Ikekawa, N. *Tetrahedron Lett.* **1984**, *25*, 4933.

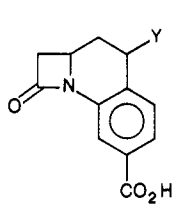
(12) Middleton, W. J. *J. Org. Chem.* **1979**, *44*, 2291.

(13) The formation of this aziridine is easily rationalized by a nucleophilic attack of sodium ethanolate on the β-lactam carbonyl followed by an intramolecular displacement.

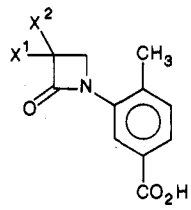
(14) Manhas, M. S.; Khatavi, M. S.; Bari, S. S.; Bose, A. K. *Tetrahedron Lett.* **1983**, *24*, 2323.

(15) Labia, R.; Andrillon, J.; Le Goffic, F. *FEBS Lett.* **1973**, *33*, 42.

Table I. Benzocarbacephems **2**, *N*-Aryl 3-Halogenated Azetidin-2-ones **3**, and Standard Compounds



2



3

no.	Y	X^1, X^2	mp, °C	$\nu_{C=O}$, cm^{-1}	K_i,[a] μM
2a	H		ref 4b	1755	20
2b[b]	Cl		ref 4b	1758	50
2c[c]	F		ref 4b	1755	39
3a[d]		F, Br	118–119	1775	60
3b		F, F	173	1790	83
3c[d]		H, Br	171	1765	40
3d		Br, Br	168	1785	160
3e[d]		H, F	173	1770	20
3f		H, H	ref 3	1750	10
calvulanic acid					0.8[e]
sulbactam					0.8[f]
cloxacillin					13[g]
penicillin G					20[h]

[a] Competitive inhibition of the TEM-1 β-lactamase (standard deviation close to 10%). [b] Mixture of two diastereoisomers. [c] Axial fluorine diastereoisomer. [d] Racemic mixture. [e] k_{inact}: 26×10^{-3} s^{-1}. [f] k_{inact}: 0.8×10^{-3} s^{-1}. [g] Poor substrate, V_{max} 2% of that of penicillin G. [h] Good substrate, K_m value.

sulbactam, whereas a moderate hydrolysis of cloxacillin was detected (V_{max} 2% of that of penicillin G). Then, the compounds were tested as inhibitors and inactivators of the enzyme, following the recommendation of Bush and Sykes.[16]

Most all of the tested compounds demonstrated a noticeable affinity for this enzyme (Table I). Compounds of the series **2** and **3** did not demonstrate any time-dependent inactivation of the β-lactamase.

Discussion

The cleavage of the β-lactam ring is a necessary condition for the use of an azetidinone as a core structure in the design of new "suicide" substrates of β-lactamases. Recently we reported the synthesis of the benzocarbacephems **2**, the cyclized analogues of the monocyclic azetidinones of type **1** which display inhibitory activities against a series of β-lactamases. These tricyclic azetidinones **2** are also competitive inhibitors of the TEM-1 β-lactamase (vide infra), but the increase of the reactivity expected from the additional ring strain does not seem sufficient to allow enzymatic ring opening. Therefore we proposed the synthesis of the 3-mono- and 3,3-dihalogenated derivatives of azetidinones **1**.

The Wasserman procedure of cyclization of β-bromopropionamides proved to be efficient also for the cyclization of substituted propionanilides. The observed $\nu_{C=O}$ IR frequencies (1765–1790 cm^{-1}) of the obtained 3-halogenated azetidinones **3** indicates a rather high potential reactivity of the β-lactam nucleus. However, compounds **3** behave as competitive inhibitors of the TEM-1 β-lactamase (Table I) and not as substrates of the enzyme. The unsubstituted compound **3f** has the best affinity for the enzyme (K_i = 10 μM) and in this aspect compares favorably with cloxacillin, which also acts as a reversible inhibitor. The suicide inhibitors, clavulanic acid and sulbactam, display better affinities, the first step in the time-dependent inhibitory process (Table I). The replacement of a single H by F has a moderate effect on the affinity whereas a more drastic effect is observed for the difluoro compound. The replacement of a single H by Br also has a moderate effect, and the F, Br compound has similar properties. The dibromo compound has only a poor affinity. There is no apparent correlation between affinity (K_i) and polarization of the β-lactam carbonyl ($\nu_{C=O}$; Table I).

In the tricyclic series **2**, the unsubstituted compound **2a** has the best affinity for the enzyme: K_i = 20 μM, a value close to that of cloxacillin. Increasing the size of the substituent Y has only a moderate unfavorable effect; from Y = H to Y = Cl the affinity decreases only by a factor of 2.5. None of the compounds of series **2** and **3** demonstrated significant time-dependent inactivation of the TEM-1 β-lactamase.

The absence of enzymatic ring opening of β-lactams **2** and **3**, even in the case of the weakly hindered and highly activated azetidinones **3b** and **3e**, suggests a nonproductive binding as a possible explanation. Extensive structure–activity relationships of β-lactam antibiotics, including theoretical and physicochemical considerations, have been investigated.[5e,17] The orientations of the carboxyl group and its distance from the β-lactam nucleus seem crucial factors to receptor recognition.[5g,18] Because of the structural and functional homologies between penicillin-sensitive D-alanyl carboxypeptidase–transpeptidase and β-lactamase active sites,[19] these parameters may also be considered with respect to the β-lactamases.[20] Both the goodness of fit to the active site and a sufficient chemical reactivity of the β-lactam nucleus seem necessary for activity against these two types of enzymes.[21,22] The binding of azetidinones **1–3** could be governed by the interaction of their aromatic carboxylic group with a basic residue, possibly Lys 73 (preprotein numbering), of the active site. Whatever their intrinsic reactivity, the binding of these relatively rigid molecules may occur in such a way that the β-lactam carbonyl is located slightly too far away from the catalytic serine hydroxyl function no. 70 for the enzymatic reaction to take place.

Experimental Section

Chemistry. Melting points were determined on a Mettler FP61 apparatus and are uncorrected. Infrared spectra were determined on a Perkin-Elmer Model 1420 spectrophotometer, and NMR spectra were recorded on a WH 90 MS apparatus (90 MHz) or a Varian EM360L NMR spectrometer equipped with a proton (60 MHz)/fluorine (56.4 MHz) probe. Mass spectra were determined on a Kratos MS50 instrument.

Ethyl 3-Bromo-2,2-difluoropropanoate (4). Ethyl bromopyruvate (9.75 g, 0.05 mol), dichloromethane (30 mL), and water (0.6 mL) were introduced in a 0.3L Monel autoclave. When closed, the autoclave was charged with sulfur tetrafluoride (19 g, 0.175 mol, 3.5 equiv). The mixture was stirred at room temperature for 18 h; then excess sulfur tetrafluoride was removed by bubbling an inert gas. The resulting mixture was added onto cooled water

(16) Bush, K.; Sykes, R. B. *Antimicrob. Agents Chemother.* **1986**, *30*, 6.
(17) Boyd, D. B. In *Chemistry and Biology of beta-lactam Antibiotics*; Morin, R. B., Gorman, M., Eds.; Academic: New York, 1982; Vol. 1, p 437.
(18) Cohen, N. C. *J. Med. Chem.* **1983**, *26*, 259.
(19) (a) Kelly, J. A.; Dideberg, O.; Charlier, P.; Wery, J. P.; Libert, M.; Moews, P. C.; Knox, J. R.; Duez, C.; Fraipont, C.; Joris, B.; Dusart, J.; Frère, J. M.; Ghuysen, J. M. *Science (Washington, D.C.)* **1986**, *231*, 1429. (b) Samraoui, B.; Sutton, B. J.; Todd, R. J.; Artymiuk, P. J.; Waley, S. G.; Phillips, D. C. *Nature (London)* **1986**, *320*, 378.
(20) Govardhan, C. P.; Pratt, R. F. *Biochemistry* **1987**, *26*, 3355.
(21) Frère, J. M.; Kelly, J. A.; Klein, D.; Ghuysen, J. M. *Biochem. J.* **1982**, *203*, 223.
(22) Cohen, N. C.; Ernest, I.; Fritz, H.; Fuhrer, H.; Rihs, G.; Scartazzini, R.; Wirz, P. *Helv. Chim. Acta* **1987**, *70*, 1967.

(100 mL) and extracted with diethyl ether (3 × 60 mL). The organic extract was washed with aqueous sodium hydrogen carbonate solution (30 mL) and brine (100 mL) and dried with sodium sulfate. The solvents were evaporated under vacuum (60 mmHg), and the residue was distilled to give 6.5 g of **4** as a colorless liquid: IR ($CHCl_3$) 1780 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) 1.4 (t, 3 H, J = 7 Hz), 3.8 (t, 2 H, J = 13.5 Hz), 4.45 ppm (q, 2 H, J = 7 Hz); ^{19}F NMR ($CDCl_3$, $CFCl_3$) −104.5 ppm (t, J = 13.5 Hz). Anal. ($C_5H_7BrF_2O_2$) C, H.

3-Bromo-2,2-difluoropropanoyl Chloride (5b). A mixture of ethyl 3-bromo-2,2-difluoropropanoate (3.26 g, 0.015 mol), phthaloyl chloride (3.05 g, 0.015 mol), and chlorosulfonic acid (1.75 g, 0.015 mol) was heated at 170 °C in a vacuum distillation apparatus equipped with a Vigreux column and a dry ice–acetone cooled receiver. Heating was maintained for about 1 h, then the apparatus was carefully connected with vacuum (120 mmHg), and products were distilled from the reaction mixture. Further distillation at atmospheric pressure of the 65–80 °C fraction gave 2 g (64%) of **5b** as a colorless liquid: bp 116–118 °C (760 mg); IR ($CHCl_3$) 1812 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) 3.85 ppm (t, J = 13 Hz); ^{19}F NMR ($CDCl_3$, $CFCl_3$) −99.5 ppm (t, J = 13 Hz); HRMS calcd for $C_3H_2BrClF_2O$ 205.8945, found 205.8951.

Preparation of the Anilides 7. General Procedure. A mixture of dry 2,6-dimethylpyridine (0.2 mL, 1.8 mmol) and *tert*-butyl 3-amino-4-methylbenzoate (**6**)[1] (372 mg, 1.8 mmol) in toluene (5 mL) was added dropwise to a stirred solution of the propanoyl chloride **5** (1.8 mmol) in toluene (2 mL). Stirring was maintained for 30 min at 20 °C. The 2,6-dimethylpyridinium chloride was filtrated and the filtrate evaporated under vacuum. The residue was purified by flash chromatography on silica gel with ethyl acetate–pentane (1:9) as eluent to give **7**.

***N*-[3-(*tert*-Butoxycarbonyl)-6-methylphenyl]-2,3-dibromo-2-fluoropropanoic acid amide (7a):** 74% yield; white solid; mp 104 °C; IR (CH_2Cl_2) 3460, 1725 cm^{-1}; ^1H NMR ($CDCl_3$) 1.63 (s, 9 H), 2.41 (s, 3 H), 4.14 (dd, 1 H, J = 8 Hz, 11.5 Hz), 4.47 (dd, 1 H, J = 11.5 Hz, 31 Hz), 7.26–8.27 ppm (m, 3 H_{arom} and NH); ^{19}F NMR ($CDCl_3$) −121 ppm (ddd, J = 5 Hz, 8 Hz, 30.4 Hz). Anal. ($C_{15}H_{18}Br_2FNO_3$) C, H.

***N*-[3-(*tert*-Butoxycarbonyl)-6-methylphenyl]-3-bromo-2,2-difluoropropanoic acid amide (7b):** 83% yield; colorless liquid; IR (CH_2Cl_2) 3420, 1710 cm^{-1}; ^1H NMR ($CDCl_3$) 1.55 (s, 9 H), 2.32 (s, 3 H), 3.83 (t, 2 H, J = 13.5 Hz), 7.2–8.2 ppm (m, 3 H_{arom} and NH); ^{19}F NMR ($CDCl_3$) −104 ppm (t, J = 13.6 Hz). Anal. ($C_{15}H_{18}BrF_2NO_3$) C, H, Br, N.

***N*-[3-(*tert*-Butoxycarbonyl)-6-methylphenyl]-2,3-dibromopropanoic acid amide (7c):** 49% yield; white solid; mp 126 °C; IR (CH_2Cl_2) 3400, 1710, 1690 cm^{-1}; ^1H NMR ($CDCl_3$) 1.57 (s, 9 H), 2.33 (s, 3 H), 3.96 (m, 2 H), 4.68 (dd, 1 H, J = 5.5 Hz, 6.8 Hz), 7.15–8.13 ppm (m, 3 H_{arom} and NH); HRMS calcd for $C_{15}H_{19}Br_2NO_3$ 420.9713, found 420.9710.

***N*-[3-(*tert*-Butoxycarbonyl)-6-methylphenyl]-2-bromo-2-propenoic Acid Amide (8).** In the chromatography of the experiment described above, an oil was eluted before **7c** and was identified as pure acrylamide 8: 29% yield; IR (CH_2Cl_2) 1720, 1688, 1608 cm^{-1}; ^1H NMR ($CDCl_3$) 1.51 (s, 9 H), 2.26 (s, 3 H), 6.02 (d, 1 H, J = 1.5 Hz), 7.01 (d, 1 H, J = 1.5 Hz), 7.06–8.27 ppm (m, 3 H_{arom}); HRMS calcd for $C_{15}H_{18}BrNO_3$ 339.0470, found 339.0468.

***N*-[3-(*tert*-Butoxycarbonyl)-6-methylphenyl]-3,2,2-tribromopropanoic Acid Amide (7d).** Bromine (32 mg, 0.4 mmol) was added to a solution of acrylamide 8 (119 mg; 0.35 mmol) and hydroquinone (2 mg) in methylene chloride (1 mL), and the reaction mixture was stirred for 2 h. After the mixture was washed with aqueous sodium hydrogen sulfite and brine, the resultant solution was dried over magnesium sulfate and passed through a pad of silica gel with dichloromethane as eluent to give 116 mg (66%) of **7d** as a colorless oil: IR (CH_2Cl_2) 3520, 1710 cm^{-1}; ^1H NMR ($CDCl_3$) 1.57 (s, 9 H), 2.37 (s, 3 H), 4.41 (s, 2 H), 7.17–8.42 ppm (m, 3 H_{arom} and NH); HRMS calcd for $C_{15}H_{18}Br_3NO_3$ 496.8838, found 496.884.

Cyclization of the 3-Bromopropionanilides 7 To Give the *N*-Arylazetidinones 9. General Procedure. Sodium hydride (640 mg, 60% dispersion in oil, 16 mmol) was washed three times with pentane under nitrogen, suspended and stirred in a mixture of dimethylformamide (7 mL) and dichloromethane (54 mL) at room temperature. Then anilide **7** (4 mmol) in the same solvent mixture (61 mL) was slowly added (2 h). After being stirred for a further 2 h, the reaction mixture was quickly washed with aqueous ammonium chloride till neutral pH. After drying on sodium sulfate, the organic phase was concentrated under reduced pressure. Flash chromatography (ether–pentane) of the residue provided the pure azetidinone **9**.

***N*-[3-(*tert*-Butoxycarbonyl)-6-methylphenyl]-3-bromo-3-fluoroazetidin-2-one (9a):** 78% yield; colorless liquid; IR (CH_2Cl_2) 1775, 1700 cm^{-1}; ^1H NMR ($CDCl_3$) 1.58 (s, 9 H), 2.41 (s, 3 H), 4.31 (dd, 1 H, J = 6.5 Hz, 18 Hz), 4.39 (dd, 1 H, J = 7.5 Hz, 18 Hz), 7.21–7.82 ppm (m, 3 H); ^{19}F NMR ($CDCl_3$) −121 ppm (dd, J = 6 Hz, 8 Hz). Anal. ($C_{15}H_{17}BrFNO_3$) C, H.

***N*-[3-(*tert*-Butoxycarbonyl)-6-methylphenyl]-3,3-difluoroazetidin-2-one (9b):** 72% yield; colorless oil; IR (CH_2Cl_2) 1780, 1700 cm^{-1}; ^1H NMR ($CDCl_3$) 1.58 (s, 9 H), 2.41 (s, 3 H), 4.2 (t, 2 H, J = 6.2 Hz), 7.22–7.82 ppm (m, 3 H); ^{19}F NMR ($CDCl_3$) −115 (t, J = 6.3 Hz). Anal. ($C_{15}H_{17}F_2NO_3$) C, H, F, N.

***N*-[3-(*tert*-Butoxycarbonyl)-6-methylphenyl]-3-bromoazetidin-2-one (9c):** 24% yield; colorless oil; IR (CH_2Cl_2) 1760, 1700 cm^{-1}; ^1H NMR ($CDCl_3$) 1.56 (s, 9 H), 2.4 (s, 3 H), 3.87 (dd, 1 H, J = 2 Hz, 6.5 Hz), 4.25 (dd, 1 H, J = 5 Hz, 6.5 Hz), 4.9 (dd, 1 H, J = 2 Hz, 5 Hz), 7.17–7.73 ppm (m, 3 H). Anal. ($C_{15}H_{18}BrNO_3$) C, H, Br, N.

***N*-[3-(*tert*-Butoxycarbonyl)-6-methylphenyl]-3,3-dibromoazetidin-2-one (9d):** 52% yield; white solid; mp 82 °C; IR (CH_2Cl_2) 1775–1705 cm^{-1}; ^1H NMR ($CDCl_3$) 1.6 (s, 9 H), 2.42 (s, 3 H), 4.53 (s, 2 H), 7.2–7.82 ppm (m, 3 H); HRMS calcd for $C_{15}H_{17}Br_2NO_3$ 420.9535, found 420.953.

***N*-[3-(*tert*-Butoxycarbonyl)-6-methylphenyl]-3-fluoroazetidin-2-one (9e).** Tri-*n*-butyltin hydride (115 mg, 0.39 mmol) and 2,2′-azobis(2-methylpropionitrile) (2 mg) were added to 3-bromo-3-fluoroazetidinone **9a** (118 mg, 0.33 mmol) in toluene (2 mL), and then the mixture was stirred at 60 °C for 90 min. The solvent was evaporated under reduced pressure, and the residue was chromatographed on silica gel with ether–pentane as eluent to give 92 mg (99%) of **9e** as a colorless liquid: IR (CH_2Cl_2) 1760, 1700 cm^{-1}; ^1H NMR ($CDCl_3$) 1.62 (s, 9 H), 2.43 (s, 3 H), 4.03 (m, 2 H), 5.71 (ddd, 1 H, J = 2.5 Hz, 3.5 Hz, 54 Hz), 7.22–7.82 ppm (m, 3 H); ^{19}F NMR ($CDCl_3$) −193 ppm (ddd, J = 9 Hz, 10 Hz, 54 Hz). Anal. ($C_{15}H_{18}FNO_3$) C, H.

Ethyl 1-[3-(*tert*-Butoxycarbonyl)-6-methylphenyl]-2-aziridinecarboxylate (10): colorless liquid; IR (CH_2Cl_2) 1740, 1705 cm^{-1}; ^1H NMR ($CDCl_3$) 1.33 (t, 3 H, J = 7 Hz), 1.59 (s, 9 H), 2.35 (s, 3 H), 2.37 (m, 1 H), 2.69 (m, 2 H), (4.27 q, 2 H, J = 7 Hz), 7.06–7.55 ppm (m, 3 H); HRMS calcd for $C_{17}H_{23}NO_4$ 305.1627, found 305.1629.

Cleavage of the *tert*-Butyl Esters To Give the Free Carboxylic Acids 3. General Procedure. To a solution of the *tert*-butyl ester **9** (110 mg, 0.37 mmol) in dichloromethane (0.5 mL) was added trifluoroacetic acid (0.5 mL) at −12 °C. Then, the mixture was allowed to warm to 10 °C during 1 h and evaporated under reduced pressure. The residue was triturated in dry ether and the acid **3** isolated by filtration.

***N*-(3-Carboxy-6-methylphenyl)-3-bromo-3-fluoroazetidin-2-one (3a):** 79% yield; white solid; IR (KBr) 1775, 1680 cm^{-1}; ^1H NMR (D_2O) 2.4 (s, 3 H), 4.62 (dd, 1 H, J = 8.5 Hz, 21.5 Hz), 4.71 (dd, 1 H, J = 6.8 Hz, 21.5 Hz), 7.36–7.82 ppm (m, 3 H); ^{19}F NMR (D_2O) −122 ppm (dd, J = 6.8 Hz, 8.5 Hz). Anal. ($C_{11}H_9BrFNO_3$) C, H.

***N*-(3-Carboxy-6-methylphenyl)-3,3-difluoroazetidin-2-one (3b):** 85% yield; white solid; IR (KBr) 1790, 1780, 1675 cm^{-1}; ^1H NMR (D_2O) 2.33 (s, 3 H), 4.42 (t, 2 H, J = 6.5 Hz), 7.25–7.72 ppm (m, 3 H); ^{19}F NMR (D_2O) −116 ppm (t, J = 6 Hz). Anal. ($C_{11}H_9F_2NO_3$) C, H, F, N.

***N*-(3-Carboxy-6-methylphenyl)-3-bromoazetidin-2-one (3c):** 92% yield; white solid; IR (KBr) 1765, 1680 cm^{-1}; ^1H NMR (D_2O) 2.36 (s, 3 H), 4.05 (dd, 1 H, J = 2 Hz, 7 Hz), 4.42 (dd, 1 H, J = 4.8 Hz, 7 Hz), 5.19 (dd, 1 H, J = 2 Hz, 4.8 Hz), 7.27–7.72 ppm (m, 3 H). Anal. ($C_{11}H_{10}BrNO_3$) C, H, Br, N.

***N*-(3-Carboxy-6-methylphenyl)-3,3-dibromoazetidin-2-one (3d):** 38% yield; white solid; mp 168 °C; IR (KBr) 1785, 1685 cm^{-1}; ^1H NMR (D_2O) 2.38 (s, 3 H), 4.54 (s, 2 H), 7.33–7.78 (m, 3 H); HRMS calcd for $C_{11}H_9Br_2NO_3$ 360.8949, found 360.895.

***N*-(3-Carboxy-6-methylphenyl)-3-fluoroazetidin-2-one (3e):** 90% yield; white solid; IR (KBr) 1770, 1675 cm^{-1}; ^1H NMR (D_2O) 2.40 (s, 3 H), 4.19 (m, 2 H), 5.89 (ddd, 1 H, J = 2.5 Hz, 3.5 Hz, 54 Hz), 7.35–7.8 ppm (m, 3 H); ^{19}F NMR (D_2O) −195 ppm (ddd,

J = 9 Hz, 11 Hz, 55 Hz). Anal. ($C_{11}H_{10}FNO_3$) C, H.

β-Lactamase Assay. Determination of Kinetic Constants. The Michaelis–Menten constants K_m and V_{max} were determined by computerized microacidimetry. The experimental conditions were pH 7 and 37 °C. Penicillin G was used as a reference antibiotic, and values for the tested compounds were expressed relative to the hydrolysis of penicillin G.

Determination of Values for K_i. For the compounds that demonstrated little or no hydrolysis by the β-lactamase, we studied the inhibition constant K_i by use of competition procedures. This study required an analysis of the rate of hydrolysis of a given substrate (e.g., penicillin G) in the presence of various concentrations of the compound, which thus behaved as an inhibitor. When inhibition of substrate hydrolysis was competitive and reversible, the presence of the inhibitor resulted in a reduced rate of substrate hydrolysis. This new rate remained essentially constant during a time when the concentration of substrate could be considered quite constant. The irreversible phase of inactivation was then studied as a function of time, i.e., the enzymatic activity was monitored after various periods of incubation of the enzyme with the inactivator at concentrations ranging from 1 to 100 μM.

Registry No. (±)-**2a**, 111773-40-1; (±)-*cis*-**2b**, 111773-41-2; (±)-*trans*-**2b**, 111773-43-4; (±)-**2c**, 111773-42-3; (±)-**3a**, 111773-35-4; **3b**, 111773-36-5; (±)-**3c**, 111773-37-6; **3d**, 111773-38-7; (±)-**3e**, 111773-39-8; **4**, 111773-24-1; (±)-**5a**, 111773-26-3; **5b**, 61444-65-3; (±)-**5** ($X^1 = X^2 = Br$, $X^3 = Cl$), 111773-27-4; **6**, 111773-25-2; (±)-**7a**, 111773-28-5; **7b**, 111793-83-0; (±)-**7c**, 111773-29-6; **7d**, 111773-31-0; **8**, 111773-30-9; (±)-**9a**, 111773-32-1; **9b**, 111773-33-2; (±)-**9c**, 111793-84-1; **9d**, 111793-85-2; (±)-**9e**, 111793-86-3; (±)-**10**, 111773-34-3; $BrCH_2COCO_2Et$, 70-23-5; β-lactamase, 9073-60-3.

Investigation of the Structural Parameters Involved in the μ and δ Opioid Receptor Discrimination of Linear Enkephalin-Related Peptides[1]

G. Gacel, J. M. Zajac, P. Delay-Goyet, V. Daugé, and B. P. Roques*

Département de Chimie Organique, U 266 INSERM, UA 498 CNRS, Faculté de Pharmacie, 75970 Paris Cedex 06, France.
Received March 6, 1987

The previous rules proposed for selective recognition of μ and δ opioid receptors by modified enkephalins were investigated through an extensive structure–activity study. Thus, modifications of the sequence of TRIMU 4 (Tyr-D-Ala-Gly-$NHCH(CH_3)CH_2CH(CH_3)_2$, a peptide that exhibits μ selectivity close to that of DAGO (Tyr-D-Ala-Gly-N(Me)Phe-Gly-ol), were performed for two positions, 2 and 4, critical for μ recognition. The drastic loss of potency following introduction of L-Ala or Aib in position 2 emphasizes the importance of the stereochemistry and the steric size of the X^2 amino acid for optimal μ binding. The enhancement of the intrinsic flexibility of the C-terminal alkyl chain of TRIMU 4 through removal of a methyl group leads to TRIMU 5 (Tyr-D-Ala-Gly-$NHCH_2CH_2CH(CH_3)_2$), a peptide with a μ selectivity similar to that of DAGO. In contrast, introduction of an *O-tert*-butyl Ser^2 residue increases affinity for δ receptors. In the hexapeptide series derived from DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr), a D-Thr^2 moiety was shown to be very efficient in improving δ recognition and δ selectivity appeared also to be modulated by the nature of the sixth residue. The potencies of the 24 peptides studied to inhibit the electrically evoked contractions of the GPI or MVD are relatively well correlated with their affinities for brain μ or δ receptors labeled with [^{3}H]DAGO or [^{3}H]DSLET, respectively. Moreover, the analgesic potency (hot plate test) of the peptides is related to their affinity for rat brain μ receptors. The wide range of receptor affinities exhibited by the compounds reported here could be useful to study the physiological role of μ and δ receptors.

The multiplicity of pharmacological effects following opioid administration (analgesia, respiratory modification, euphoria, addiction) could be related to the nondiscriminate interaction of these compounds with several types of receptors.[2] This assumption is supported by the existence of different binding sites for morphine and the endogenous morphine-like peptides.[3] Indeed, it is now well established that [Met]- and [Leu]enkephalin interact in the central nervous system, as well as in peripheral organs, with at least two types of receptors designated μ and δ.[3] Among various physiological functions, the μ type seems to be preferentially involved in pain control at the supraspinal level[4-8] while the δ type may be especially implicated in the behavioral effects of opioids.[9-12] Nevertheless, unambiguous elucidation of the physiological role of each receptor type requires ideally the use of completely selective effectors (agonists and antagonists).

The dual interaction of enkephalins with two distinct binding sites means that, as classical neurotransmitters, the endogenous opioid peptides are able to adopt different conformations at different receptor sites.[13] This is con-

(1) Symbols and abbreviations are in accordance with recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature: *Biochem. J.* **1984**, *219*, 345. The following other abbreviations have been used: FAB, fast atom bombardment; THF, tetrahydrofuran; MeOH, methanol; $CHCl_3$, chloroform; EtOAc, ethyl acetate; Et_2O, ether; DCC, cyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; Aib, 2-aminoisobutyric acid; Pen, penicillamine; Az-Gly, $NH_2NHCOOH$; [Met]enkephalin, Tyr-Gly-Gly-Phe-Met; [Leu]enkephalin, Tyr-Gly-Gly-Phe-Leu; Metkephamid, Tyr-D-Ala-Gly-Phe-N(Me)-Met-NH_2.

(2) Martin, W. R.; Eades, C. G.; Thompson, J. A.; Huppler, R. E.; Gilbert, P. E. *J. Pharmacol. Exp. Ther.* **1976**, *197*, 517.

(3) Lord, J. A. H.; Waterfield, A. A.; Hughes, J.; Kosterlitz, H. W. *Nature (London)* **1977**, *267*, 495.

(4) Herz, A.; Bläsig, J.; Emrich, H. M.; Cording, C.; Pirée, S.; Kölling, A.; Zerssen, D. V. In *The Endorphins, Advances in Biochemical Psychopharmacology*; Costa, E., Trabucchi, M., Eds.; Raven: New York, 1978; Vol. 18, p 333.

(5) Gacel, G.; Fourniê-Zaluski, M.-C.; Fellion, E.; Roques, B. P. *J. Med. Chem.* **1981**, *24*, 1119.

(6) Chang, K. J.; Cuatrecasas, P.; Wei, E. T.; Chang, J. K. *Life Sci.* **1982**, *30*, 1547.

(7) Chaillet, P.; Coulaud, A.; Zajac, J.-M.; Fourniê-Zaluski, M.-C.; Costentin, J.; Roques, B. P. *Eur. J. Pharmacol.* **1984**, *101*, 83.

(8) Fang, F. G.; Fields, H. L.; Lee, N. M. *J. Pharmacol. Exp. Ther.* **1986**, *238*, 1039.

(9) Stein, L.; Belluzzi, J. D. In *The Endorphins, Advances in Biochemical Psychopharmacology*; Costa, E., Trabucchi, M., Eds.; Raven: New York, 1978; Vol. 18, p 299.

(10) Clouet, D. *Ann. N.Y. Acad. Sci.* **1982**, *398*, 130.

(11) Roques, B. P. *J. Pharmacol.* **1985**, *16*, 5.

(12) Roques, B. P.; Daugé, V.; Gacel, G.; Fourniê-Zaluski, M.-C. In *Biological Psychiatry 1985*; Shagass, C., Josiassen, R. C., Bridger, W. H., Weiss, K. J., Stoff, D., Simpson, G. M., Eds.; Elsevier: Amsterdam, 1986; Vol. 7, p 297.

0022-2623/88/1831-0374$01.50/0