Journal of Industrial Microbiology (1995) 15, 108–111

Microbial transformation of *N*-heptyl physostigmine, a semisynthetic alkaloid inhibitor of cholinesterase

L So[1], R White[1], B Arison[1], B Petuch[1], MS Schwartz[2], H Cheng[2], R Monaghan[1] and TS Chen[1]

[1]*Merck Research Laboratories, Department of Fermentation Microbiology, Rahway, NJ 07065;* [2]*Department of Drug Metabolism, West Point, PA 19486, USA*

The microbiological transformation of *N*-heptyl physostigmine (L-693,487) (1), a semisynthetic physostigmine cholinesterase inhibitor, was investigated using *Verticillium lecanii* MF 5713 (ATCC 74148), *Acremonium* sp MF 5723 (ATCC 74164) and *Actinoplanes* sp MA 6559 (ATCC 53771). Nine microbial metabolites (2–10) of 1 were isolated and purified using reversed-phase HPLC. The structures of the metabolites were established using spectroscopic techniques including MS and NMR. Some of the microbial metabolites were identical to metabolites present in urine of a dog treated with 1.

Keywords: biotransformation; *N*-heptyl physostigmine; cholinesterase inhibitors

Introduction

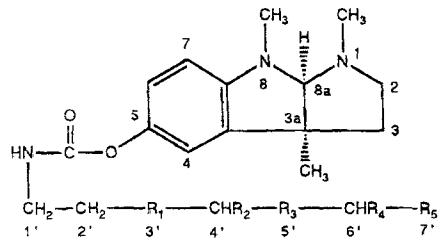
Physostigmine is one of the oldest known alkaloids isolated from Calabar beans [5] and has also been obtained from the culture filtrate of *Streptomyces pseudogriseolus* subsp *iriomotensis* subsp nov [4]. Physostigmine is a cholinesterase inhibitor and certain anticholinesterase drugs have been suggested to be useful in the treatment of Alzheimer's disease [1]. The use of physostigmine is considerably limited by its short half-life of effect, poor oral bioavailability, and severe dose-limiting side-effects particularly towards the digestive system.

N-heptyl physostigmine (structure **1**, Figure 1) [2] is a new semisynthetic carbamate derivative of physostigmine. Compound **1** is a potent reversible cholinesterase inhibitor, has a long duration of action and crosses the blood–brain barrier readily. Studies on microbial transformation of the compound were initiated in the hope that mammalian metabolites could be produced microbiologically and obtained in quantities sufficient for further pharmacological and toxicological studies. The present communication describes the microbial oxidation of **1** by *Verticillium lecanii* MF 5713, *Acremonium* sp MF 5723 and *Actinoplanes* sp MA 6559.

Materials and methods

General

All organic solvents (EM Science, Gibbstown, NJ, USA) were HPLC grade. Water was purified in a Millipore Milli-Q system (Bedford, MA, USA). *N*-heptyl physostigmine (**1**) was prepared at Merck Research Laboratories (Rahway, NJ, USA). ^{1}H NMR experiments were performed on either a Varian Unity 400 or a VXR-500 NMR spectrometer and chemical shifts were referenced to CD_2HOD solvent peak ($\delta_H = 3.30$). FABMS measurements were obtained on a Finnigan Mat TSQ 70 instrument using glycerol as a matrix.



	R_1	R_2	R_3	R_4	R_5
L-693,487 (1)	CH_2	H	CH_2	H	CH_3
2	CHOH	H	CH_2	H	CH_3
3	CH_2	OH	CH_2	H	CH_3
4	CH_2	H	CHOH	H	CH_3
5	CH_2	H	CH_2	OH	CH_3
6	C=O	H	CH_2	H	CH_3
7	C=O	OH	CH_2	H	CH_3
8	CHOH	OH	CH_2	H	CH_3
9	CH_2	H	CH_2	H	COOH
10	CH_2	H	COOH	--	--

Figure 1 The structure of *N*-heptyl-physostigmine and the metabolites

Production of metabolites

Frozen vials (2.0 ml) of cultures were used to inoculate 250-ml 3-baffled Erlenmeyer shake flasks containing 50 ml of an autoclaved seed medium consisting of (g L^{-1}): dex-

Correspondence: TS Chen PhD, Department 807, Building R80Y-205, Merck Research Laboratories, Rahway, NJ 07065, USA
Received 25 January 1995; accepted 6 April 1995

trin 10.0, glucose 1.0, beef extract 3.0, Ardamine PH (Yeast Products, Inc, Clifton, NJ, USA) 5.0, N-Z Amine type E 5.0, $MgSO_4 \cdot 7H_2O$ 0.05, K_2HPO_4 0.37, and $CaCO_3$ 0.5. The pH of the seed medium was adjusted to 7.1 with 1 N NaOH before autoclaving it. The seed flask was incubated on a rotary shaker (220 rpm) at 27° C for 24 h. A 2.5-ml aliquot of the developed seed was used to inoculate a 250-ml non-baffled Erlenmeyer shake flask containing 50 ml of production medium. Production medium for *Verticillium lecanii* MF 5713 and *Acremonium* sp MF 5723 consisted of (g L^{-1}): glucose 20.0, soybean meal 5.0, yeast extract 5.0, NaCl 5.0. pH was adjusted to 7.0 before autoclaving. Production medium for *Actinoplanes* sp MA 6559 consisted of (g L^{-1}): glucose 10.0, Hycase SF 2.0, Beef extract 1.0, Corn steep liquor 3.0. pH was adjusted to 7.0 before autoclaving. *N*-Heptylphysostigmine (**1**) was added as a solution in methylsulfoxide 24 h after incubation to achieve a final concentration of 0.05 mg ml^{-1}. The shake flasks were subsequently incubated at 27° C in the dark on a rotary shaker. The fermentation broth was examined for the presence of metabolites by HPLC. Analytical HPLC was performed by a Zorbax RX C-8, 4.6 mm × 25-cm column (Zorbax, Chadde Ford, PA, USA) and monitored at 240 nm. The column was developed at 1.0 ml min^{-1} using a 35-min linear gradient from 15% to 80% acetonitrile in water containing 0.1% H_3PO_4 (v/v).

Isolation and purification of metabolites **2–10**

Metabolites **5** and **9** from *Verticillium lecanii* MF 5713: The whole fermentation broth (150 ml) was centrifuged at 3000 rpm for 10 min and the aqueous phase was adjusted to pH 9.0. It was then extracted with CH_2Cl_2 (1:1). The aqueous phase, after CH_2Cl_2 extraction, was passed through a Spe-ed octadecyl cartridge (14% carbon load, Applied Separation) under vacuum. The cartridge was washed with 50 ml H_2O and eluted with 150 ml MeOH. Both the CH_2Cl_2 extract and MeOH eluant were evaporated to dryness under reduced pressure. The resulting oils were dissolved in MeOH and subjected to HPLC purification. Preparative HPLC was carried out on a Zorbax RX-C8 column (9.4 mm ID × 25 cm) at room temperature and the effluent was monitored at 240 nm. For the metabolite obtained by solid-phase extraction, the column was developed at 3 ml min^{-1} with a linear gradient from 10% to 50% CH_3CN in 0.1% trifluoroacetic acid (TFA) over 35 min. The compound was collected during repeated injections of the above extract. The fractions at retention time 21 min were pooled and evaporated to dryness to yield 6.0 mg of metabolite **9**. For the CH_2Cl_2 extract, the column was developed at 3 ml min^{-1} with a linear gradient from 10% to 50% CH_3CN in 0.1% TFA over 55 min. The compound was collected during repeated injection of the above extract. Fractions at retention time 29 min were pooled and evaporated to dryness to yield 0.8 mg of metabolite **5**.

Metabolites **5** and **10** from *Acremonium* sp MF 5723: The whole broth (150 ml) was processed as described above to yield 0.8 mg of metabolite **10** and 4.0 mg of metabolite **5**.

Metabolites **2**, **3**, **4**, **5**, **6**, **7**, and **8** from *Actinoplanes* sp MA 6559: All the metabolites produced by *Actinoplanes* sp MA 6559 were extracted into CH_2Cl_2. CH_2Cl_2 was evaporated to dryness and the resulting oil was dissolved in MeOH. HPLC was carried out on a Zorbax RX-C8 column (9.4 mm × 25 cm) developed at 3 ml min^{-1} with a linear solvent gradient obtained by mixing a 0.1% aqueous TFA solution and CH_3CN. The initial ratio 90:10 was increased to 30:70 over 80 min. Fractions of retention time at 41, 49, 57, 59, 61, 68, and 75 min were pooled and evaporated to dryness to yield 0.4 mg of metabolite **8**, 0.5 mg of metabolite **7**, 0.25 mg of metabolite **5**, 0.5 mg of metabolite **4**, 0.7 mg of metabolite **3**, 1.0 mg of metabolite **2**, and 0.35 mg of metabolite **6**, respectively.

Assay of acetylcholinesterase activity

The acetylcholinesterase inhibition assay was a modification of the method of C Johnson *et al* [6]. Test compounds were initially dissolved in CH_3CN or MeOH to a concentration of 1 mg ml^{-1} and further diluted with acetonitrile to concentrations of 0.1 to 25 μg ml^{-1}. Samples (2.5 μl) of these solutions were added to 250 μl of 0.1% BSA (Sigma, St Louis, MO, USA) in 0.1% Triton X-100 (Surfact-Amps X-100, Pierce, Rockford, IL, USA). Two 90-μl aliquots of the diluted solutions were mixed with 10 μl human type XIII acetylcholinesterase (Sigma #C-5400, 0.1 unit ml^{-1} 0.1% BSA in 0.1% Triton X-100) in mini scintillation vials. After a 2-h pre-incubation at room temperature, 25 μl of 0.25 M potassium phosphate buffer (pH 7.4) and 20 μl of ^3H substrate solution were added. The substrate solution consisted of 5 μCi ml^{-1} ^3H acetylcholine (New England Nuclear #NET–113, Boston, MA, USA) in 0.5 mM acetylcholine chloride (Sigma). After 30 min at room temperature, the reaction was quenched by the addition of 100 ml of a stopping solution containing 1 M chloroacetic acid, 0.5 M NaOH, and 2 M NaCl. Four milliliters of a toluene-based scintillation fluid containing 10% isoamyl alcohol were added and the mini-vials were counted in a liquid scintillation counter (Beckman 6000SC, Beckman, Fullerton, CA, USA) for 2 min each. IC_{50} concentrations were calculated using Beckman Immuno-Fit EIA/RIA software (% enzyme activity vs log standard concentration).

Results and discussion

Biotransformation

Microbial transformation has been known as an excellent tool of xenobiotic metabolism to mimic drug metabolism in mammals because hydroxylations of most drugs by a range of microorganisms mechanistically resemble the same reactions catalyzed by hepatic cytochrome P-450 monooxygenases [7]. Additionally, and perhaps the most attractive feature of microbial transformations, is the facile scale-up of desired biotransformations to yield quantities of metabolites for biological evaluation. Our goal was to use the microbial transformation approach to produce mammalian metabolites of the drug for biological testing and, hopefully, to provide second generation drugs. Thirty microorganisms were screened for their ability to metabolize *N*-heptyl physostigmine (**1**). HPLC chromatographic analysis of the culture extracts indicated that three microorganisms were capable of carrying out transformation of this compound.

Table 1 ^{1}H NMR data of *N*-heptyl physostigmine (**1**) and microbial transformation products (**2–10**)

Proton position	Data for compound no									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
4	6.96 (d, 2.1 Hz)	6.79 (d, 2.0 Hz)	6.73 (d, 2.0 Hz)	6.73 (d, 2.0 Hz)	6.96 (d, 2 Hz)	6.73 (s)	6.73 (d, 2 Hz)	6.73 (d, 2 Hz)	6.95 (d, 2.0 Hz)	6.96 (d, 2.0 Hz)
6	6.94 (dd, 8.1, 2.2 Hz)	6.79 (dd, 8.0, 2.2 Hz)	6.75 (dd, 8.0, 2.1 Hz)	6.75 (dd, 7.0, 2.1 Hz)	6.92 (dd, 8.1, 2 Hz)	6.74 (dd, 8.0, 2 Hz)	6.75 (dd, 8.0, 2 Hz)	6.75 (dd, 8.0, 2 Hz)	6.93 (dd, 8.0, 2.1 Hz)	6.89 (dd, 8.0, 2.1 Hz)
7	6.62 (d, 8.1 Hz)	6.46 (d, 8.1 Hz)	6.40 (d, 8.0 Hz)	6.40 (d, 8.0 Hz)	6.69 (d, 8.0 Hz)	6.39 (d, 8.1 Hz)	6.40 (d, 8.0 Hz)	6.40 (d, 8.1 Hz)	6.65 (d, 8.0 Hz)	6.65 (d, 8.1 Hz)
8a	4.93 (s)	4.27 (s)	4.05 (s)	4.06 (s)	4.07 (s)	4.05 (s)	4.06 (s)	4.05 (s)	4.92 (s)	4.89 (s)
2	2.70 (m), 2.60 (m)	2.86 (m), 2.70 (m)	2.70 (m), 2.59 (m)	2.70 (m), 2.60 (m)	2.70, 2.60 (m)	2.59, 2.51 (m)	2.70, 2.60 (m)	2.70, 2.60 (m)	3.37 (m)	3.32 (m)
1′	3.17 (t, 7.0 Hz)	3.26 (m)	3.16 (m)	3.16 (m)	3.16 (t, 7.1 Hz)	3.38 (t, 7.2 Hz)	3.40 (m)	3.30 (m)	3.16 (bt)	3.18 (bt)
8-NCH3	3.12 (s)	2.99 (s)	2.94 (s)	2.94 (s)	2.94 (s)	2.94 (s)	2.94 (s)	2.94 (s)	3.14 (s)	3.30 (s)
1-NCH3	2.80 (s)	2.60 (s)	2.51 (s)	2.51 (s)	2.50 (s)	2.51 (s)	2.51 (s)	2.51 (s)	2.92 (s)	3.12 (s)
3	2.30 (m)	2.05 (m)	1.96 (m)	1.97 (m)	1.98 (m)	1.96 (m)	1.96 (m)	1.96 (m)	2.39 (m)	2.30 (m)
6′	1.35 (m)	1.34 (m)	1.35–1.58 (m)	1.41 (m)	3.72 (m)	1.32 (m)	1.45 (m)	1.51, 1.42 (m)	2.29 (bt)	–
5′	1.35 (m)	1.34 (m)	1.35–1.58 (m)	3.45 (m)	1.40 (m)	1.55 (m)	1.70, 1.55 (m)	1.51, 1.42 (m)	1.63 (m)	–
2′	1.57 (m)	1.58 (m), 1.72 (m)	1.68, 1.35–1.58 (m)	1.51, 1.41 (m)	1.56, 1.40 (m)	2.71 (m)	2.83 (m)	1.76, 1.65 (m)	1.57 (m)	1.65 (m)
3a-CH3	1.48 (s)	1.43 (s)	1.40 (s)	1.41 (s)	1.40 (s)	1.40 (s)	1.40 (s)	1.40 (s)	1.50 (s)	1.50 (s)
3′	1.35 (m)	3.61 (m)	1.35–1.58 (m)	1.41 (m)	1.35–1.45 (m)	–	–	3.50 (m)	1.38 (m)	1.60 (m)
4′	1.35 (m)	1.46 (m)	3.56 (m)	1.41–1.3 (m)	1.35–1.45 (m)	2.48 (t, 7.8 Hz)	4.08 (dd, 5, 3 Hz)	3.42 (m)	1.38 (m)	2.34 (bt)
7′	0.90 (t, 7.0 Hz)	0.93 (t, 7.0 Hz)	0.93 (t, 7.0 Hz)	0.94 (t, 7.0 Hz)	1.15 (d, 5.2 Hz)	0.92 (t, 7.1 Hz)	0.95 (t, 7.2 Hz)	0.95 (t, 7.0 Hz)	–	–

^{1}H NMR was recorded at 400 MHz in CD_3OD; δ_H (multiplicity, J = Hz)

Table 2 Acetylcholinesterase activity of *N*-heptyl physostigmine (**1**) and its biotransformation derivatives (**2**–**10**)

1 and derivatives	IC_{50} (ng ml^{-1})
L-693,487 (**1**)	0.2
2	12.5
3	1.7
4	4.0
5	2.4
6	0.9
7	2.74
8	28.6
9	16.3
10	125

Structure determination

Incubation of *Verticillium lecanii* MF 5713 with **1** gave metabolites **9** and **5**. The positive FAB-MS of metabolite **9** gave an $[M+H]^+$ at m/z 390 which corresponds to an increase of 30 mass units from heptyl physostigmine, indicating an oxidation product. ^1H NMR analysis of **9** indicated that the terminal methyl on the heptyl side chain had been oxidized to a carboxyl group. Oxidation was evident from the absence of the terminal methyl triplet at 0.91 ppm and the appearance of a new methylene signal at 2.30 ppm, typical of a CH_2 adjacent to a carbonyl. The positive FAB-MS of metabolite **5** gave $[M+H]^+$ at m/z 376, an increase of 16 mass units, indicating a monohydroxylated derivative. The ^1H NMR spectrum showed that the terminal methyl group C-7′ of the heptyl side chain, observed as a triplet at 0.95 ppm in the NMR spectrum of **1**, appeared as doublet at 1.15 ppm and a new one-proton quartet was observed at 3.72 ppm. The ^1H NMR in conjunction with MS data confirmed that **5** was hydroxylated on the penultimate methylene carbon (C-6′) of the heptyl side chain.

Preparative incubation of **1** with *Acremonium* sp MF 5723 also gave two metabolites. One was a C-6′ hydroxylated derivative **5** based on MS and NMR comparison with the same material isolated from an incubation with *Verticillium lecanii* MF 5713. The ^1H NMR analysis of the second metabolite, **10**, indicated that the heptyl side chain had been reduced to five carbons with the terminal carbon oxidized to a carboxyl. Key features were the presence of only four methylenes that could be associated with the side chain with the chemical shift of one at 2.35 ppm typical of a CH_2 adjacent to a carboxyl group. A molecular ion at m/z 362 $[M+H]^+$ in the positive FAB-MS of **10** confirmed the structure. It appears that metabolite **10** is formed by terminal oxidation of the heptyl side chain followed by β-oxidation.

Incubation with *Actinoplanes* sp MA 6559 for 6 h after addition of **1** gave seven metabolites. One of these was determined to be **5** by ^1H NMR and MS described above. Positive ion FAB-MS gave $[M+H]^+$ signals at 376 for the microbial transformation metabolites **2**, **3**, and **4**, indicating that they were mono-hydroxylated derivatives. These compounds were identified as monohydroxylated at C-3′, C-4′, and C-5′, respectively, based on unambiguous ^1H signal assignment by ^1H-^1H decoupling experiments (Table 1). Positive ion FAB-MS gave $[M+H]^+$ at m/z 374 for microbial transformation metabolite **6**, suggesting it to be a keto derivative. The presence of a C-3′ keto in metabolite **6** was established by assignment of two CH_2 proton triplet signals at d2.71 (H-2′) and 2.48 ppm (H-4′) typical of CH_2 adjacent to a carbonyl. Metabolite **6** was further hydroxylated at C-4′ to give the C-3′ keto and C-4′ hydroxyl derivative **7**. Compared with **6**, the ^1H NMR of **7** showed the absence of H-6′ and the appearance of one new proton at 4.08 ppm consistent with a -CHOH-group. Observation of a molecular ion at m/z 390 $[M+H]^+$ in the positive FAB-MS further supported the assignment. Positive FAB-MS of metabolite **8** gave an $[M+H]^+$ signal at m/z 392, suggesting that it was a di-hydroxylated derivative. The final structure identification was based on unambiguous ^1H assignments. The signals of H-3′ and H-4′ were assigned to 3.50 and 3.42 ppm, respectively.

Biological activity

Data on inhibition of acetylcholinesterase by the microbial metabolites of **1** are summarized in Table 2. The monohydroxylated and keto derivatives at the heptyl side chain maintained good biological activity. Dihydroxylated and carboxylic acid derivatives reduced the activity dramatically.

Hichens *et al* [3] have reported that the metabolic products of hydroxylation, terminal oxidation, and terminal oxidation plus β oxidation are present in the urine of dogs treated with **1**. Our observations reinforce the concepts that microorganisms can be used as predictive models for metabolic transformations in mammals [7].

Acknowledgements

We express our gratitude to Dr L Colwell and his colleagues for providing FAB-MS support.

References

1 Anonymous. 1991. Synapton promising in Alzheimer's? Script no 164322.
2 Brufani M, C Castellano, M Marta, A Oliverio, F Pavone and M Pomponi. 1989. Physostigmine derivatives with acetylcholinesterase inhibition properties, and manufacture. US Patent no 4831155 (CA 110: 146935v).
3 Hichens M, HGP Kari and KP Vyas. 1993. Hexahydropyrrolo[2,3-B]indole derivatives. US Patent no 5206260 (CA 119: 66604g).
4 Iwasa T, S Harada and Y Sato. 1981. Miticidal antibiotics C-8030 B, C, D and physostigmine produced by *Streptomyces pseudogriseolus* subsp *iriomotensis* subsp nov. J Takeda Res Lab 40: 1226.
5 Jobst J and O Hesse. 1964. Ueber die bohne von Calabar. Liebigs Ann 129: 115–121.
6 Johnson CD and RL Russell. 1975. A rapid, simple radiometric assay for cholinesterase, suitable for multiple determinations. Analyt Biochem 64: 229–238.
7 Smith RV and JP Rosazza. 1975. Microbial models of mammalian metabolism. J Pharm Sci 64: 1737–1759.