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(*S*)-NORCOCLAURINE IS THE CENTRAL INTERMEDIATE IN BENZYLISOQUINOLINE ALKALOID BIOSYNTHESIS

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Key Word Index—*Berberis stolonifera*, *Peumus boldus*, *Eschscholtzia californica*, *Argemone hispida*, cell suspension cultures, norcoclaurine, benzylisoquinoline alkaloid biosynthesis

Abstract—Feeding experiments with (*S*)-[1-^{13}C]-norcoclaurine demonstrate that this trihydroxylated precursor is specifically incorporated into protoberberine, aporphine and benzophenanthridine alkaloids in cell suspension cultures, as well as into pavine and benzophenanthridine alkaloids in whole plants The rates of incorporation ranged from 2 5 to 36% This reveals that tyrosine is metabolized to dopamine and *p*-hydroxyphenylacetaldehyde which then condense to form norcoclaurine, thus explaining the lack of incorporation of DOPA or dopamine into the benzylic portion of reticuline derived alkaloids.

INTRODUCTION

Numerous tracer experiments point to the fact that both C_6–C_2 units comprising the benzylisoquinoline skeleton are derived from tyrosine [1]. Norlaudanosoline (**1**) has been predicted [2, 3] to be the central intermediate for a multitude of isoquinoline alkaloids, a hypothesis which was later based on tracer experiments involving specifically labelled norlaudanosoline [4, 5]. The metabolites dopamine, DOPA and dopamine derived 3,4-dihydroxyphenylacetaldehyde have always been assumed as logic-intermediates in the tyrosine–norlaudanosoline pathway. However, incorporation of labelled DOPA or dopamine into the alkaloids in question showed that only the isoquinoline and not the benzylic portions are labelled [see ref. 1]. These experiments also proved that the two C_6–C_2 units derived from tyrosine differ from one another

Recently, we resolved this discrepancy with feeding experiments, involving both tritiated and ^{14}C labelled precursors, which demonstrated that both coclaurine as well as reticuline (**2**) have one and the same biogenetic origin [6] This central intermediate was proposed by us to be norcoclaurine (**3**, demethylcoclaurine, higenamine) Indeed, application of this trihydroxylated compound to *Papaver somniferum* seedlings led to specific incorporation into thebaine [7]. However, in several other plant species feeding led to browning of the leaf or stem, thereby inactivating these metabolites as potential precursors

To circumvent this problem and to establish the role of norcoclaurine (**3**) in benzylisoquinoline biosynthesis, we fed enantiomerically pure (*R*)- and (*S*)-[1-^{13}C]-norcoclaurine to high alkaloid producing plant cell suspension cultures. Application of this methodology proved beyond doubt that (*S*)-norcoclaurine is indeed the precursor of different biogenetic types of reticuline derived alkaloids

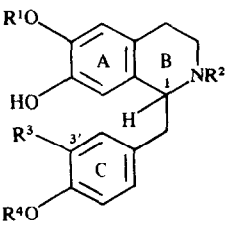
RESULTS AND DISCUSSION

(*R*)- and (*S*)-[1-^{13}C]-norcoclaurine were synthesized and fed to cell suspension cultures of *Berberis stolonifera*, which are a fruitful source of protoberberines [8]. Application of even substantial amounts of norcoclaurine (0 5 mM) to the growth medium did not induce discoloration (browning) of the culture, which was probably due to both the slightly acidic pH of the medium and the rapid uptake of the precursor.

The crude alkaloidal extract was subjected to ^{13}C NMR analysis, and exhibited isotopic enrichment in the downfield region of the spectrum (δ 140.09 and 139.9) These enhanced resonances could be attributed to the two major protoberberine alkaloids of *Berberis stolonifera*, namely jatrorrhizine and columbamine, respectively.

An excerpt of the proton-decoupled ^{13}C NMR spectrum of (*S*)-[1-^{13}C]-norcoclaurine labelled columbamine is given in Fig. 1. The carbon atom at C-14 is distinctly enriched, proving a specific and unambiguous incorporation of (*S*)-norcoclaurine into the protoberberine class of alkaloids. A 19 5% ^{13}C-atom excess was determined by EIMS

We also investigated the incorporation of (*S*)-norcoclaurine into a pavine-type compound. Cell cultures



1 $R^1 = R^2 = R^4 = H$, $R^3 = OH$
2 $R^1 = R^2 = R^4 = Me$, $R^3 = OH$
3 $R^1 = R^2 = R^3 = R^4 = H$

1-H = α = (*S*), 1-H = β = (*R*)

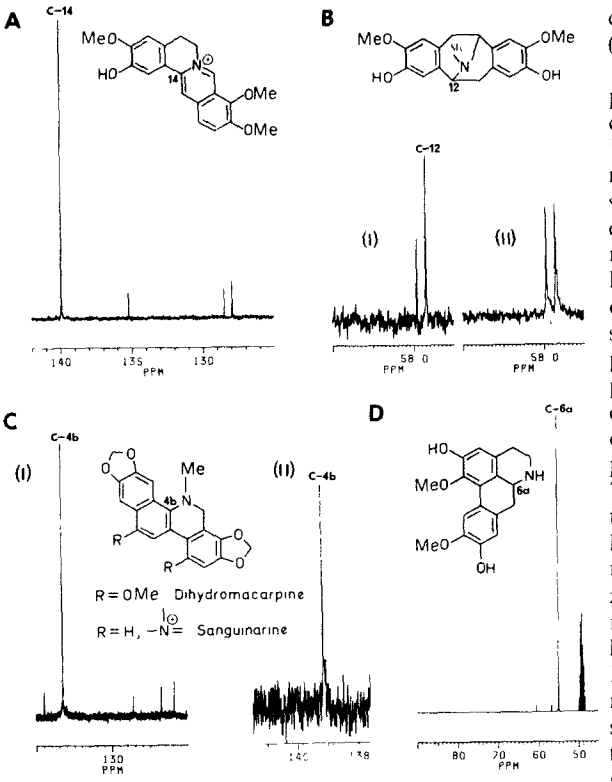


Fig 1 Incorporation of (*S*)-norcoclaurine into reticuline-derived benzylisoquinoline alkaloids

of *Argemone hispida* could be established, but unfortunately these did not produce the desired alkaloid bisnorargemonine Therefore, incorporation into whole plants using the wick method was attempted Bisnorargemonine was isolated, rigorously purified and the ^{13}C NMR spectra recorded There is a definite incorporation of the isotopically labelled C-1 of norcoclaurine corresponding to C-12 of bisnorargemonine (δ57 68) (Fig 1B I) An enrichment factor of 2 5% ^{13}C-atom excess was determined, a satisfactory result in whole plant systems As a comparison, the partial ^{13}C NMR spectrum of natural bisnorargemonine depicting the resonances of C-12 and C-6 is shown in Fig 1B II

After attempts at feeding *Eschscholtzia californica* plants through the root system resulted in a blackening of the tissue, (*S*)-norcoclaurine was fed to *E californica* cell suspension cultures and found to specifically label the benzophenanthridine alkaloid dihydromacarpine [9] (Fig. 1C I, δ136 44) An enrichment factor of 10 5% ^{13}C-atom excess was determined by EIMS In contrast to the *E californica* system, application of norcoclaurine to roots of whole plants of *Macleaya cordata* resulted in rapid uptake and incorporation into the benzophenanthridine alkaloid, sanguinarine (Fig. 1C II) Finally, a rapidly growing cell culture of *Peumus boldus*, shown to produce the aporphine norboldine (laurolitsine) as one of the major alkaloids, was fed in a manner analogous to the other cell culture experiments

The extremely high and specific incorporation of (*S*)-norcoclaurine is portrayed in Fig 1D The enlarged signal at δ54 81 corresponds to C-6a of norboldine, confirming unequivocal labelling of the target compound (36 0% ^{13}C-atom excess)

To conclusively examine the sterospecificity of incorporation we applied the chiral (*R*)-counterparts of norcoclaurine and coclaurine, labelled in both the ^{13}C and ^{14}C form Absolutely no incorporation of the (*R*)-metabolites into the alkaloids under question was observed This again proves that exclusively the (*S*)-enantiomer of norcoclaurine is the true precursor of (*S*)-reticuline, which is the central intermediate of the alkaloids investigated here The results revealed in this paper demonstrate beyond doubt that (*S*)-norcoclaurine is specifically incorporated into the corresponding sites of protoberberine, aporphine, benzophenanthridine and pavine alkaloids without scrambling of the label to other carbons The recording high rates of incorporation preclude the channelling of a side metabolite into a main pathway This observation supports our previous view [6, 7], that not norlaudanosoline but rather norcoclaurine is the true first alkaloidal intermediate in benzylisoquinoline formation in plants In view of the recent observation that animals and man are also able to produce benzylisoquinoline alkaloids of the morphinan type [10, 11], it will be of interest to investigate whether or not norcoclaurine is the central intermediate in these systems too Having established the role of norcoclaurine in plant metabolism we will attempt to clarify the later biosynthetic events on its way to reticuline The main open features in the pathway leading to reticuline are the sequence of steps of *O*- as well as *N*-methylation and the additional hydroxylation of the C-ring Considering the fundamental role of norcoclaurine, the applicable enzymes which have already been discovered and characterized [12, 13] need to be renamed The previously assumed divergence from norlaudanosoline has led, for instance in the case of the enzyme which condenses dopamine with the C_6–C_2-aldehyde, to the designation 'norlaudanosoline'-synthase [14] In spite of the fact that both aldehydic intermediates 3,4-dihydroxyphenylacetaldehyde and 4-hydroxyphenylacetaldehyde (the now established true reactant) have approximately the same K_M-values for both aldehydes In the light of these recent findings, this enzyme has to be renamed norcoclaurine-synthase

The fact that norlaudanosoline [4, 5] and several intermediates of the reticuline pathway [1] are incorporated *in vivo* and *in vitro* into metabolites which potentially can be incorporated into given target molecules like reticuline, shows that the enzymes of the latter pathway are relatively unspecific, as compared to the biosynthesis of more sophisticated structures which are dependent on highly specific enzyme activity

EXPERIMENTAL

General Mps uncorr ^1H NMR spectra 360 MHz, using TMS as an int standard ^{13}C NMR spectra were recorded at 90 MHz, with the solvent as the int standard All chemical shifts in δ, solvents as specified

For 2-D HETCOR and COLOC NMR spectra, typically 512 FID's, data matrix 512 × 1024 points, zero filling to 1024 × 2048 points EIMS (70 eV) were recorded with a Finnigan MAT 44S spectrometer Optical rotations were measured with a Perkin-Elmer 241 polarimeter at 20 Thin layer chromatography was conducted using silica gel 60 F_{254} precoated plates 0 5 mm (Merck), unless otherwise specified

Plant materials. All plant cell cultures were provided by our cell culture laboratory They were grown in Linsmaier–Skoog medium [15] Typically, cultures were in 11 Erlenmeyer flasks containing 250 ml of medium Filter sterilized (*R*)- or (*S*)-[1-^{13}C]-norcoclaurine solution (final conc 0 5 mM) was added at day 3 of growth to the cultures, which were then allowed to grow for another 5 days, after which period the cells were harvested and the alkaloids in question isolated *A hispida* was grown outdoors from seeds The labelled norcoclaurine was applied by the cotton wick method A total of 3 mg in 0 5 ml H_2O was supplied to each plant Typically, three plants were allowed to metabolize for 4 days after which time the plants were depotted and extracted *Macleaya cordata* plants were likewise grown outdoors from seeds Norcoclaurine was supplied through the root system Forty-eight plantlets 2–5 months old were each administered 0 5 mg norcoclaurine in 400 μl H_2O Metabolism was allowed to proceed for 2 days whereupon the plantlets were extracted exhaustively in boiling 80% MeOH

Isolation of alkaloids Columbamine Ca 80 g fr wt cells were suspended in 250 ml MeOH and disintegrated by disruption with an Ultraturrax blendor at room temp The resulting extract was filtered and applied to a Servachrome XAD 300–500 μ column (2 5 × 10 cm) on which the alkaloids were absorbed The column was washed with H_2O and subsequently the alkaloids desorbed with MeOH The alkaloids were purified by prep TLC (a) and (b) on silica gel 60 F_{254} plates, (c) on Al_2O_3 sheets with the following solvent systems (a) CH_2Cl_2–MeOH–NH_4OH (90 9 1), (b) $CHCl_3$–MeOH–NH_4OH (340 90·3), (c) cyclohexane–$CHCl_3$–HOAc (9 9 2) R_f-columbamine was 0 10, 0 18 and 0 33, respectively Pure columbamine (8 mg) was isolated ^{13}C NMR signals were assigned with the aid of C, H-heteronuclear correlated 2D-NMR spectra, namely HETCOR (C–H correlation for direct C–H connectivities) and COLOC for correlated long-range couplings EIMS *m/z* (rel int) 341 $[M]^+$ (24), 176 (13), 164 (88), 149 (100), 135 (19) ^{1}H NMR (CD_3OD) δ3 30 (2H, *t*, H-5), 3.89 (3H, *s*, 3-OMe), 4 04 (3H, *s*, 10-OMe), 4 17 (3H, *s*, 9-OMe), 4 93 (2H, *t*, H-6), 6 92 (1H, *s*, H-4), 7 48 (1H, *s*, H-1), 7 93 (1H, *d*, H-12), 8 01 (1H, *d*, H-11), 8 49 (1H, *s*, H-13), 9 69 (1H, *s*, H-8) ^{13}C NMR (CD_3OD) δ27 67 (C-5), 56 76 (O-3-Me), 57 49 (C-6), 57.82 (O-10-Me), 62 74 (O-9-Me), 112 23 (C-4), 113 15 (C-1), 120 48 (C-1a), 120 94 (C-13), 123 0 (C-8a), 124 66 (C-12), 127 77 (C-11), 128 42 (C-4a), 134 87 (C-12a), 139 90 (C-14), 145.29 (C-9), 146 34 (C-8), 147 92 (C-2), 151 70 (C-10), 152.16 (C-3)

Norboldine (laurolitsine) Norboldine was isolated essentially the same way as described for columbamine TLC was conducted in solvents (a) and (b) (R_f norboldine 0 18 and 0.52, respectively) 5 mg of norboldine was recovered ^{13}C NMR assignments are based on literature values [16] EIMS *m/z* (rel int) 314 $[M]^+$ (56), 313 $[M-1]^+$ (100), 298 (24), 283 (17), 282 (18), 270 (13), 254 (17) ^{1}H NMR (CD_3OD) δ2.54–2 96 (6H, *m*, H-4, H-5, H-7), 3 57 (3H, *s*, 1-OMe), 3 85 (3H, *s*, 10-Me), 6 55 (1H, *s*, H-3), 6 69 (1H, *s*, H-8), 7 97 (1H, *s*, H-11) ^{13}C NMR (CD_3OD) δ28 75 (C-4), 36.68 (C-7), 43.67 (C-5), 54.82 (C-6a), 56 55 (O-10-Me), 60 31 (O-1-Me), 112 83 (C-11), 115 5 (C-3), 115 79 (C-8), 124 76 (C-11a), 126 58 (C-1b), 127 63 (C-1a), 130 04 (C-3a), 130 29 (C-7a), 144 42 (C-1), 147 16 (C-9), 147 8 (C-10), 150 88 (C-2)

Dihydromacarpine and macarpine The filtered cells from *E californica* cultures were suspended in MeOH and refluxed for 30 min Both alkaloids were purified on a Sephadex LH-20 column (5 5 × 52 cm) and subsequently by TLC, (d) toluene–Me_2CO–EtOAc (7 2·1) macarpine (R_f 0 14) was then reduced to dihydromacarpine (BH_4^-) and rerun in solvent, (e) *n*-hexane–Et_2O (6 1), (R_f 0 28) Dihydromacarpine isolated from the culture (10 mg) and the reduced compound from the macarpine isolation (5 mg) yielded identical ^{13}C NMR and ^{1}H NMR spectra The quaternary carbons of the ^{13}C NMR spectrum were assigned with the aid of 2D-COLOC experiments. EIMS *m/z* (rel. int.) 393 $[M]^+$ (86), 378 (100), 363 (28), 348 (25), 333 (22), 197 (20) ^{1}H NMR ($CDCl_3$) δ2 52 (3H, *s*, N-Me), 3 87 (3H, *s*, 10-OMe), 3 99 (3H, *s*, 12-OMe), 4 08 (2H, *s*, H-6), 6 00 (2H, *s*, 7,8-OCH_2O), 6 04 (2H, *s*, 2,3-OCH_2O), 6 61 (1H, *s*, H-9), 7 53 (1H, *s*, H-1), 7 66 (1H, *s*, H-4), 7 82 (1H, *s*, H-11) ^{13}C NMR ($CDCl_3$) δ41 47 (N-Me), 49.89 (C-6), 56 20 (O-12-Me), 57 51 (O-10-Me), 95 75 (C-9), 99 65 (C-1), 101 28 (C-4), 102 27 (2,3-OCH_2O), 102 59 (7,8-OCH_2O), 104 79 (C-11), 114 82 (C-10a), 116 19 (C-6a), 122 32 (C-12a), 124 77 (C-10b), 127 91 (C-4a), 136 44 (C-4b), 139 82 (C-7), 148 29 (C-2), 148 53 (C-8), 149.27 (C-3), 151.87 (C-12), 153.16 (C-10)

Bisnorargemonine Three plants of *A hispida* were worked-up as follows The plant (13 8 g fr wt including the root system) was extracted with hot MeOH The extract was subjected to TLC in solvent systems (f) (cyclohexane–$CHCl_3$–diethylamine 7 2 1), (a), and (g) EtOAc–$CHCl_3$–EtOH–diethylamine (14 3.2 1) Bisnorargemonine was the major pavine alkaloid and yielded the following R_f-values (f) 0 29, (a) 0 38, (g) 0 28 A total of 4 mg of the target compound was isolated ^{13}C NMR assignments were made possible with the help of 2D-NMR experiments (HETCOR, COLOC) EIMS *m/z* (rel int) 327 $[M]^+$ (18), 312 (4), 190 (100), 175 (8), 147 (6) ^{1}H NMR (CD_3OD) δ2 36 (1H, *d*), 2 37 (3H, *s*, N-Me), 2 50 (1H, *d*), 3 20 (1H, *dd*), 3 26 (1H, *dd*), 3 67 (3H, *s*, 3-OMe), 3 75 (3H, *s*, 8-OMe), 3 83 (1H, *d*), 3 90 (1H, *d*), 6.35 (1H, *s*, H-10), 6 47 (1H, *s*, H-4), 6 55 (1H, *s*, H-1), 6 70 (1H, *s*, H-7) ^{13}C NMR (CD_3OD) δ34 28 (C-11), 34 68 (C-5), 40 64 (N-Me), 56 35 (O-3-Me), 56 47 (O-8-Me), 57 69 (C-12), 57.92 (C-6), 111 35 (C-7), 112 65 (C-4), 114 55 (C-1), 115 98 (C-10), 123 82 (C-4a), 125 16 (C-10a), 129 79 (C-6a), 131 08 (C-12a), 146 02 (C-2), 146 45 (C-9), 147 86 (C-8), 148 22 (C-3)

Sanguinarine Macleaya cordata plants (23 g fr wt) were extracted exhaustively in boiling 80% MeOH The crude extract was fractionated by TLC in the following systems (h) $CHCl_3$–hexane–MeOH (9 10 1) sanguinarine R_f 0 47 and (i) *n*-PrOH–HCO_2H–H_2O (90 1 9) sanguinarine R_f 0 22

Sanguinarine purification was achieved by repeated TLC using both solvent systems Partial assignment of the ^{13}C NMR spectrum was made by comparison to ref [17]

Synthesis of (R)- *and* (S)-[1-^{13}C]-*norcoclaurine* HBr The enantiomers of (*R*)- and (*S*)-[1-^{13}C]-coclaurine were prepared by standard procedures [18] The optically pure [1-^{13}C]-coclaurines (200 mg, 0 62 mmol) were refluxed with 47% aq HBr for 20 min The reaction mixture was cooled to room temp, diluted with EtOH and evapd under red pres (40°) The crystalline residue was washed with cold EtOH and EtOAc and then left to dry *in vacuo* (40°)

(R)-[1-^{13}C]-*Norcoclaurine hydrobromide* 202 mg, 0 58 mmol (93% yield), mp 269–270° (Lit [19] 270–272°), $\alpha_D = +27\,6°$, (MeOH, *c* 0 25)

(S)-[1-^{13}C]-*Norcoclaurine hydrobromide* 194 mg, 0 55 mmol (89% yield), mp 269–270°, $\alpha_D = -25\,4°$, (MeOH, *c* 0 25) The enantiomeric purity of each form was further checked by derivatization with the acid chloride of MTPA (α-methoxy-α-trifluoromethylphenylacetic acid) and subsequent ^{13}C NMR analysis (solvent $CDCl_3$) The (*R*)-enantiomer revealed only one enriched signal at δ52 63, whereas the (*S*)-enantiomer showed an intense resonance at 54.86, with no resonance in the 52 ppm region which could have corresponded to the (*R*)-enantiomer With this method we were able to assign an enantiomeric purity of at least 99% and probably more to our thus purified chiral norcoclaurines

(R)- *and* (S)-[1-^{13}C]-*Norcoclaurine* HCl The optically pure [1-^{13}C]-norcoclaurine hydrobromides (100 mg, 0 28 mmol) were dissolved in 20 ml H_2O–MeOH and stirred on an ice-water bath

The incubation mixture was made alkaline (pH 8.0) with aq NH_4OH and rapidly extracted with 30 ml portions of EtOAc (3 ×) The combined organic layers were dried over anhydrous Na_2SO_4 and concd *in vacuo* (40°C) The white ppt (base) was subsequently taken up in MeOH–HCl and again concd under red pres (40°) The (*R*)- and (*S*)-norcoclaurine hydrochlorides were re-crystallized from EtOH and air-dried (*R*)-[1-^{13}C]-norcoclaurine HCl 62 mg, 0.2 mmol (71%), mp 249–254° (Lit [20] 256–263°) (*S*)-[1-^{13}C]-norcoclaurine HCl 58 mg, 0.19 mmol (69%), mp 250–254 The spectroscopic data (NMR, EIMS, UV) of the enriched alkaloids were identical to authentic samples.

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REFERENCES

1 Spenser, I D (1968) in *Comprehensive Biochemistry* (Florkin, M, and Stotz, E H, eds) Chapt 20, p 231 Elsevier, Amsterdam
2 Winterstein, E and Trier, G (1910) in *Die Alkaloide*, p 307 Gebruder Borntrager, Berlin
3 Robinson, R (1917) *J Chem Soc* **111**, 876
4 Battersby, A R and Binks, R (1960) *Proc Chem Soc* 360
5 Battersby, A R, Binks, R, Francis, R J, McCaldin, D J and Ramuz, H (1964) *J Chem Soc* 3600
6 Stadler, R, Kutchan, T M, Loeffler, S, Nagakura, N, Cassels, B and Zenk, M H (1987) *Tetrahedron Letters* **28**, 1251
7 Loeffler, S, Stadler, R, Nagakura, N and Zenk, M H (1987) *J Chem Soc Chem Commun* 1160
8 Hinz, H and Zenk, M H (1981) *Naturwissenshaften* **67**, 620
9 Schumacher, H M, Gundlach, H, Fiedler, F and Zenk, M H (1987) *Plant Cell Rep* **6**, 410
10 Donnerer, J, Oka, K, Brossi, A, Rice, K C and Spector, S (1986) *Proc Natl Acad Sci U S A* **83**, 4566
11 Weitz, C J, Faull, K F and Goldstein, A (1987) *Nature* **330**, 674
12 Zenk, M H (1985) in *The Chemistry and Biology of Isoquinoline Alkaloids* (Phillipson, J D *et al*, eds) p 240 Springer, Heidelberg
13 Zenk, M H, Rueffer, M, Amann, M and Deus-Neumann, B (1985) *J Nat Prod* **48**, 725
14 Schumacher, H M, Rueffer, M, Nagakura, N and Zenk, M H (1983) *Planta Med* **48**, 212
15 Linsmaier, E M and Skoog, F (1965) *Physiol Plant* **18**, 110
16 Marsaioli, A J, Reis, F A M, Magalhaes, A F and Rúveda, E A (1979) *Phytochemistry* **18**, 165
17 Blasko, G, Cordell, G A, Bhamarapravati, S and Beecher, W W (1988) *Heterocycles* **27**, 911
18 Stadler, R, Loeffler S, Cassels, B K and Zenk, M H (1988) *Phytochemistry* **27**, 2557
19 Yamaguchi, H (1958) *Yakugaku Zasshi* **78**, 692
20 Koshiyama, H, Ohkuma, H, Kawaguchi, H, Hsu, H-Y and Chen, Y-P (1970) *Chem Pharm Bull* **18**, 2564