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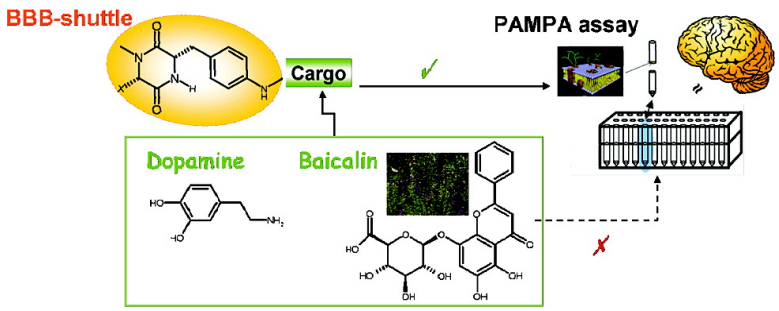

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Diketopiperazines as a Tool for the Study of Transport across the Blood−Brain Barrier (BBB) and Their Potential Use as BBB-Shuttles

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Abstract: Here we prepared and evaluated two libraries of mono-*N*-methylated and di-*N*-methylated diketopiperazines (DKPs) by parallel artificial membrane permeability assay and immobilized artificial membrane chromatography in order to obtain information on the features that govern the passage of peptidic molecules across the blood−brain barrier (BBB) by passive diffusion. On the basis of the results from these two libraries, we prepared and evaluated several DKP−baicalin and DKP−dopamine constructs. The DKPs or cyclic dipeptide scaffolds can be considered a novel family of brain delivery systems (BBB-shuttles) to transport to the brain drugs and other cargos that cannot cross the BBB unaided.

Introduction

Several severe health disorders require treatment of the brain. These include not only neurodegenerative diseases, such as Parkinson and Alzheimer disease, but also CNS[1] diseases, such as schizophrenia, epilepsy, and bipolar disorder. Cancer, HIV, and even certain aspects of obesity are also considered cerebral pharmaceutical targets. In many cases, there are promising compounds for their treatment; however, owing to blood−brain barrier (BBB) transport problems, >98% of these potential agents do not reach the drug development stage.[2]

Drug delivery to the brain is complex, as compounds must cross the BBB. This barrier is a natural defense mechanism designed to keep harmful substances out of this organ. The BBB controls the composition of brain extracellular fluid independently of fluctuations within blood. It is also impermeable to many environmental compounds and drugs. Nonbrain capillaries have paracellular pathways, while brain capillaries do not have these pathways, because of the presence of tight junctions between endothelial cells, which form a continuous membrane with no fenestrations. Therefore, possible transport mechanisms are all transcellular (Figure 1).[3−5]

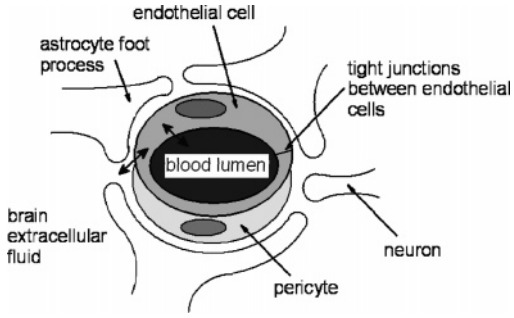

Figure 1. Anatomical basis of the blood−brain barrier. Endothelial cells of the brain capillaries form tight junctions.

Specific transporters mediate the access of certain crucial molecules for the brain, such as glucose, isolated amino acids, and ions.[6,7] Other compounds or drugs are dependent on diffusion through the lipid bilayers of endothelial membranes, which requires that these compounds exhibit a certain degree of lipophilicity.[8−10] Here we focused our attention on the passive diffusion mechanism, as it is a nonsaturable and spontaneous transport process.

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(1) Abbreviations. ACH, α-cyano-4-hydroxy-cinnamic acid; BBEC, bovine brain endothelial cells; 2-ClTrt, 2-chlorotrityl resin; Da, dalton; DBU, 1,8-diazabicyclo[5.4.0]-undec-7-en; CNS, central nervous system; DCM, dichloromethane; DEA, diethylamine; DIAD, diisopropylazodicarboxylate; DIEA, diisopropylethylamine; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DMAP, 4-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; ESI-MS, electrospray ionization-mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; ^{1}H NMR, proton nuclear magnetic resonance; HOAt, 7-aza-1-hydroxybenzotriazole; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; HPLC−MS, high performance liquid chromatography−mass spectrometry; MALDI, matrix-assisted laser desorption ionization; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; MeCN, acetonitrile; MeOH, methanol; MRI, magnetic resonance imaging; MS, mass spectrometry; NMP, *N*-methylpyrrolidone; NMR, nuclear magnetic resonance; PET, positron emission tomography; POP, prolyl oligopeptidase; PyAOP, (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; R_f, retention factor; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; THF, tetrahydrofurane; TLC, thin layer chromatography; t_R, retention time.

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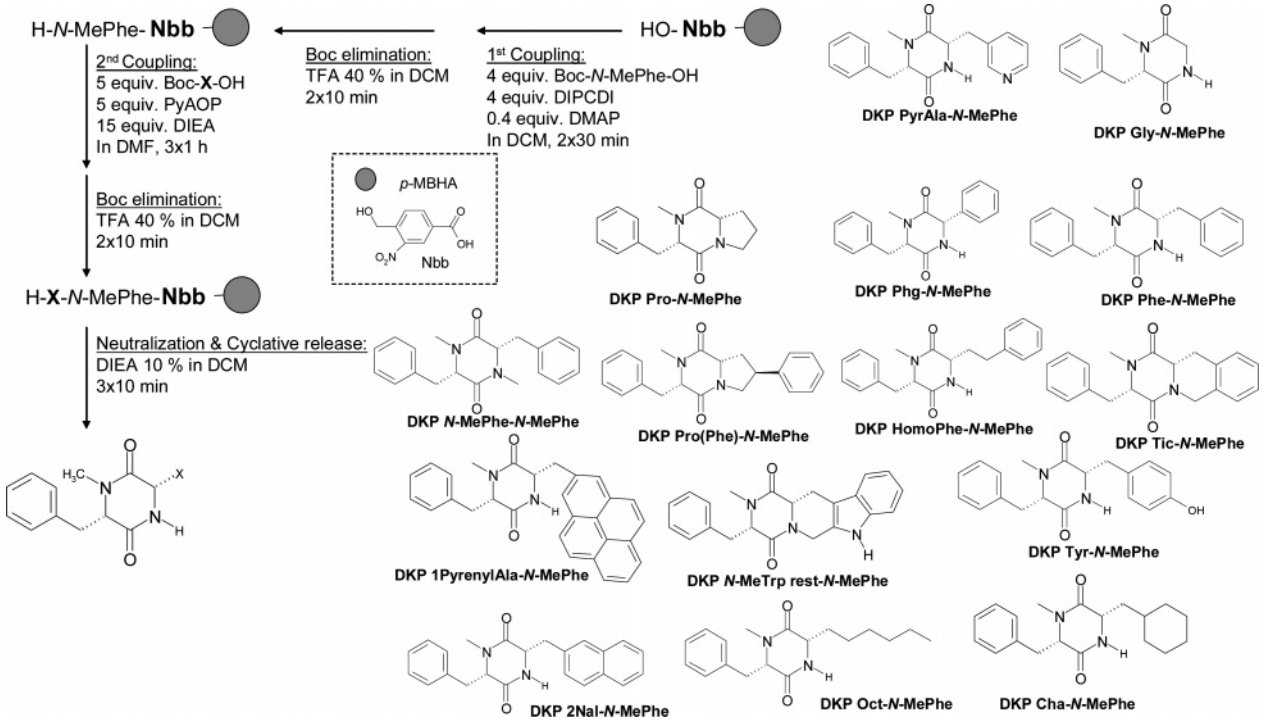
Scheme 1. Synthetic Methodology for the Preparation of the First Library of DKPs



Given that there are several promising peptide-based drugs for the treatment and diagnosis of diseases of the CNS,[11,12] information on the regulation of peptide transport through the BBB by passive diffusion is urgently required. This information will contribute to the design of novel peptidic drugs with improved transport properties and also to the design of peptidic molecules that act as BBB-shuttles for drugs that cannot cross the BBB unaided.

Diketopiperazines (DKPs) are very common in nature[13,14] and comprise a family of compounds with many potential applications of interest. Our attention was drawn to these compounds because of an unexpected observation made during a previous project performed in the field of evolutionary combinatorial chemistry.[15] During the synthesis and evaluation of several control peptides designed to optimize various parameters of an in vitro cellular assay, one of these peptides, the *N*-methylphenylalanine trimer (Ac-*N*-MePhe-*N*-MePhe-*N*-MePhe-$CONH_2$), degraded to form the DKP *N*-MePhe-*N*-MePhe, which has the capacity, by passive diffusion, to cross the BBB model of bovine brain endothelial cells (BBEC assay).

The transport properties of DKP *N*-MePhe-*N*-MePhe had never been studied, although descriptions of this compound had been reported. The pathways used to synthesize picroroccellin, the first alkaloid isolated from a lichen, *Roccella fuciformis* DC, in 1877 by Stenhouse and Groves[16] and later studied by Forster[17] and Marcuccio,[18] includes one based on the autoxidation of the DKP *N*-MePhe-*N*-MePhe. In the 1980s Stermitz[19] described a new alkaloid, 2,5-dibenzyl-1,4-dimethylpiperazine, isolated from *Zanthoxylum aff. Arborescens L*, a Mexican tree of the citrus family. In the leaves and fruits of this tree, which is commonly called "Yucu tisḿa" (herb to kill fish), a new family of alkaloids was found, which included 2,5-dibenzyl-1,4-dimethylpiperazine. This compound is biosynthetically related to the DKP Phe-Phe by *N*-methylation and reduction.

Results and Discussion

First Library of DKPs. A first library of 15 DKPs with distinct side chains was prepared using a solid-phase methodology (Scheme 1). For most cases, the only purification required was a desalting step. The side chains were chosen in order to explore the effect of adding or removing a methylene group, adding a nitrogen or oxygen atom, and adding a larger aromatic ring or a hydrophobic, but not aromatic, side chain. All DKPs in this first library were characterized by HPLC, HPLC-MS, and MALDI-TOF MS. All were obtained with purity higher than 90%, except for 1PyrenylAla-*N*-MePhe, which was purified in order to remove the salts and byproducts resulting from the use of the Fmoc derivative 1PyrenylAla in its synthesis. In the case of Phg-*N*-MePhe, two peaks were detected, corresponding to the epimerization of C_α of the phenylglycine (Phg) residue. Phg is prone to racemize during coupling.[20]

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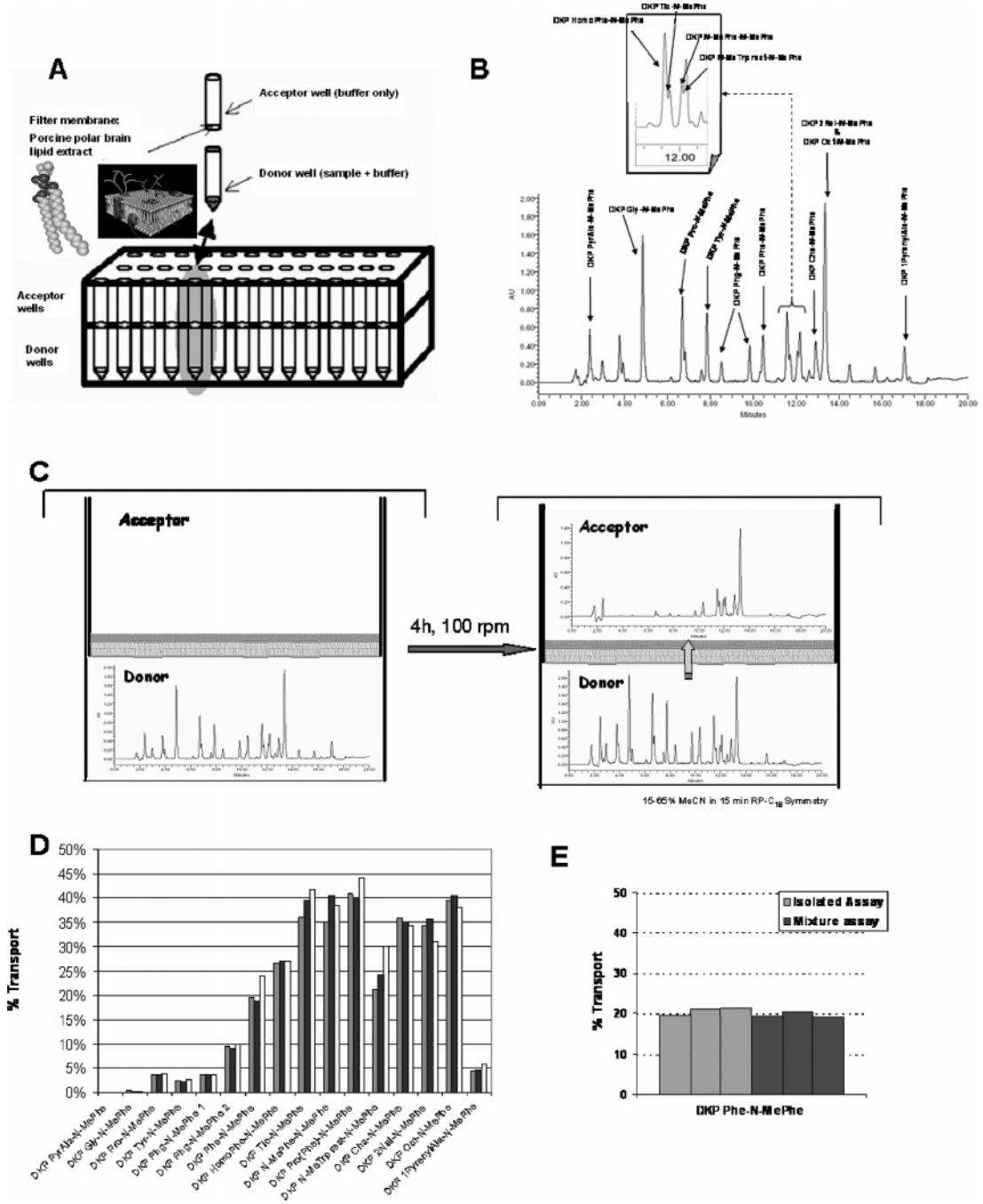

Figure 2. (a) Scheme of the PAMPA assay. (b) HPLC chromatogram from the mixture of the first library of DKPs in a linear gradient from 15 to 65% MeCN in 15 min using a Symmetry C_{18} column (150 × 4.6 mm × 5 μm, 100 Å, Waters). (c) HPLC chromatograms of donor and acceptor PAMPA compartments at time 0 and 4 h for the mixture of the first library of DKPs, in a linear gradient from 15 to 65% MeCN in 15 min using a Symmetry C_{18} column (150 × 4.6 mm × 5 μm, 100 Å, Waters). (d) Percentage of transport after 4 h in the PAMPA for the DKPs of the first library. *These compounds, overlapped in the mixture, were isolated and analyzed. (e) Comparison of the evaluation of Phe-*N*-MePhe in the mixture and as an isolated compound.

BBB Transport Evaluation of the First Library of DKPs by Parallel Artificial Membrane Permeability Assay (PAMPA). Although the ideal assay to evaluate the capacity of a compound to enter the CNS may be regarded as that which most closely resembles the in vivo situation, other aspects must be considered. In general terms, the method chosen to evaluate BBB transport should be simple, automatizable with high throughput and low cost, and require only a small amount of test compound.

Here we focused on the passive diffusion transport mechanism, a nonsaturable and spontaneous process, thereby allowing us to not be restricted to mimicking substrates of BBB transporters. For this reason we used an evaluation method, PAMPA, that predicts or evaluates only this transport mechanism.

The PAMPA assay, originally introduced by Kansy,[21] uses an artificial membrane in the form of filter-supported phospholipid bilayers. In the case of mimicking the BBB, a porcine polar lipid extract is used to coat the filter[22] (Figure 2a). The

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phospholipid membrane mimics the cell membrane but has no means for active or paracellular transport of drug molecules and is therefore a convenient tool to evaluate the transport of compounds by passive diffusion. Here we explored the possibility of evaluating mixtures of compounds by PAMPA. The unstirred water layer of the standard in vitro assay does not accurately reflect physiological conditions;[23] in vivo it is negligible, due to the small diameter of brain capillaries (~7 μm) as well as to the efficiency of mixing due to the passage of erythrocytes.[24] Therefore, to mimic the dynamism of the in vivo environment, we shook the plate at 100 rpm for 4 h. The experiment was run for a time that ensures linear transport without any back-diffusion. We recommend using these conditions, which are more restrictive than those of the standard assay, as they provide more reliable data. Last, we also evaluated control molecules with known high brain penetration capacities, propranolol (a well-known β-adrenergic receptor blocker) and carbamazepine (an anticonvulsant and mood-stabilizing drug, used primarily in the treatment of epilepsy and bipolar disorder).

The DKP compounds of the first library were evaluated as a mixture, which has the advantage of speeding up the high-throughput of the PAMPA assay and facilitating comparison between the compounds and establishment of rules.

A mixture of the 15 DKPs was prepared at a concentration of 200 μM each. The mixture was analyzed by HPLC (Figure 2b) and the PAMPA assay was performed (Figure 2c,d). As a control, the DKP Phe-*N*-MePhe was evaluated separately in another well of the same PAMPA plate. The same transport properties were found when this compound was examined alone or in a mixture (Figure 2e). The compounds that overlapped in the mixture were evaluated alone in another well of the same PAMPA plate.

From the mono-*N*-methylated DKPs in the first library, Oct-*N*-MePhe (39.4%), Cha-*N*-MePhe (35.1%), and 2Nal-*N*-MePhe (33.7%) followed by HomoPhe-*N*-MePhe (26.8%) and Phe-*N*-MePhe (20.8%) were the best in terms of percentage of transport after 4 h. *N*-MePhe-*N*-MePhe and Tic-*N*-MePhe, which are closely related to Phe-*N*-MePhe, but with an extra *N*-methyl group, showed greater transport in the PAMPA assay compared with Phe-*N*-MePhe. However, it was observed that increasing flexibility (from Phg-*N*-MePhe to Phe-*N*-MePhe and then to HomoPhe-*N*-MePhe) did not always lead to improved transport of the compounds (for example, Tic-*N*-MePhe vs *N*-MePhe-*N*-MePhe). It is also of interest to note the distinct transport capacity of the two diastereomers of Phg-*N*-MePhe. Our results indicate that the PAMPA assay can be used to evaluate the transport of mixtures of compounds by passive diffusion. Using this assay, we determined that the first library of DKPs contains compounds with permeability values close to those of propranolol (27.1%) and carbamazepine (26.4%).

Synthesis and Evaluation of a Second Library of DKPs. Not all the DKPs of the first library showed efficient transport across the BBB PAMPA model. After choosing those with the best capacity, we designed and prepared a second library, in this case di-*N*-methylated DKPs, which were also synthesized in solid-phase (Scheme 2) and evaluated by PAMPA (Table 1). The introduction of a second *N*-methyl group was done to increase the half-life of these compounds in the body, thereby avoiding degradation by peptidases in the blood or associated with cerebral microvessels.[25] Several authors[24,26−34] have proposed that the introduction of a *N*-methyl group increases BBB transport capacity. However, we wanted to check whether this second library showed improved transport capacity or whether they became too hydrophobic and were retained in the membrane.

The *N*-methyl amino acids were incorporated using commercially available building blocks or via *N*-methylation on solid-phase[35,36] using the corresponding non-*N*-methylated amino acid.

The yields of this second library were excellent, corroborating the high tendency of *N*-methyl amino acids to form DKPs. A comparison of the DKPs that had both amide bonds *N*-methylated with those that only had one *N*-methylated amide bond indicated that the presence of the second *N*-methyl group in the amide bond improves BBB transport capacity (Phe, HomoPhe) because it reduces the number of potential hydrogen bonds that the DKP can form. However, in the case of highly hydrophobic DKPs (2Nal, Cha, Oct, 1PyrenylAla), the addition of a second *N*-methyl group can be a disadvantage, because the corresponding DKP is more retained in the membrane. The positive transport measured for these compounds by the PAMPA assay as compared to the control compounds, indicated that these compounds may cross the BBB in vivo by passive diffusion. Moreover, comparison of the DKP scaffold (*N*-MePhe-*N*-MePhe) to the corresponding linear peptide (Ac-*N*-MePhe-*N*-MePhe-$CONH_2$) highlights that the DKP is a privileged scaffold in terms of BBB penetration potential.

BBB Transport Evaluation by Immobilized Artificial Membrane Chromatography (IAMC). Another technique to evaluate the passive diffusion is IAMC. This simple, fast, and inexpensive chromatographic technique was also used to evaluate our libraries (Figure 3). Developed by Pidgeon,[37] IAMC uses phospholipid molecules covalently immobilized to silica particles at high density as the stationary phase. It has been used to purify membrane proteins,[38] to immobilize enzymes,[39] and to predict transport across biological barriers.[40] IAMC exhibits a good correlation[40] with in vitro cell-based assays[41] and is convenient in terms of high throughput. IAMC interactions include ionic, lipophilic, and hydrogen-bonding interactions, which can be combined under a parameter known as phospholipophilicity.

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Scheme 2. Synthetic Methodology for the Preparation of the Library of Di-*N*-methylated DKPs

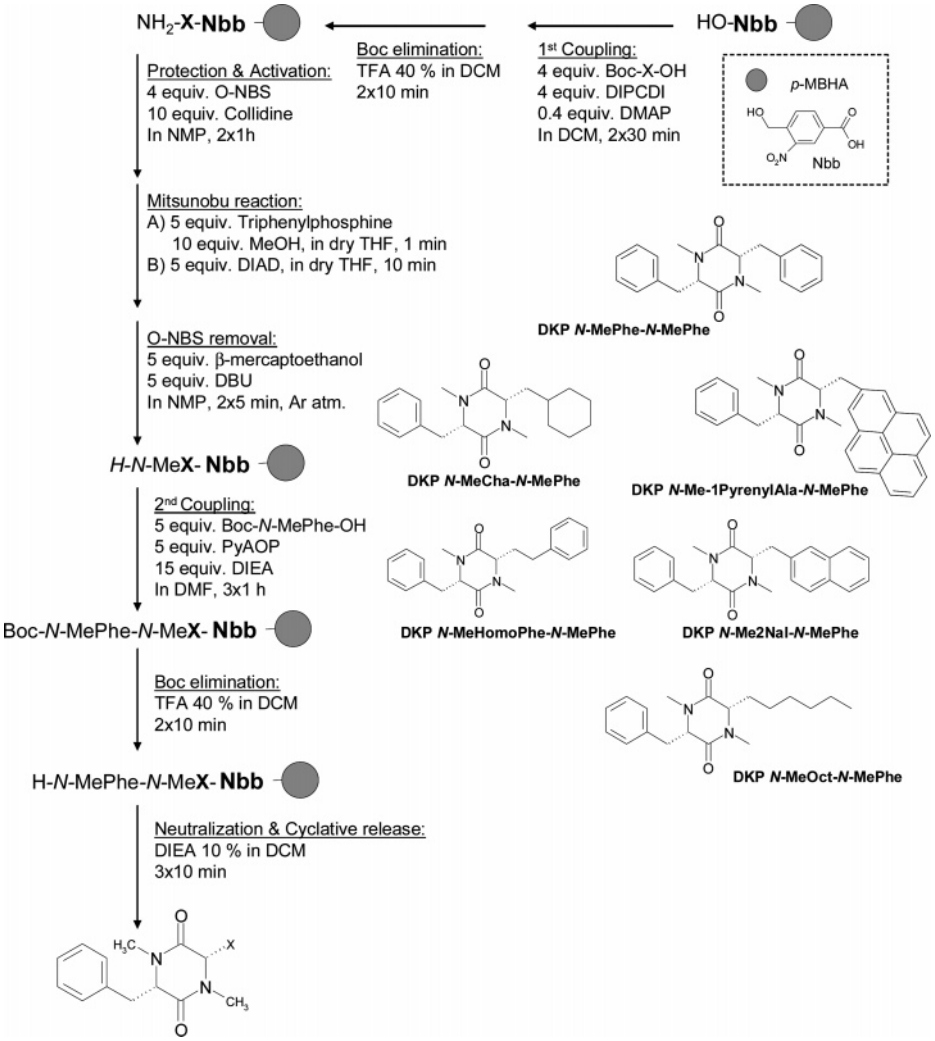


Table 1. Percentage of Transport after 4 h, Effective Permeability (P_e and log P_e), and Percentage of Retention in the Membrane in the PAMPA Assay for the DKPs X-*N*-MePhe and *N*-MePhe-*N*-MeX and Control Compounds Such as Propranolol, Carbamazepine, and the Linear Peptide Ac-*N*-MePhe-*N*-MePhe-$CONH_2$

compound	% transport at 4 h	P_e (cm/s)	log P_e	% retention in the membrane
Phe-*N*-MePhe	20.8	1.28×10^{-5}	−4.9	3
HomoPhe-*N*-MePhe	26.8	1.82×10^{-5}	−4.7	0
2Nal-*N*-MePhe	33.7	2.65×10^{-5}	−4.6	3
Cha-*N*-MePhe	35.1	2.87×10^{-5}	−4.5	4
Oct-*N*-MePhe	39.4	3.68×10^{-5}	−4.4	1
1PyrenylAla-*N*-MePhe	5.0	0.12×10^{-5}	−5.9	76
N-MePhe-*N*-MePhe	37.4	3.40×10^{-5}	−4.5	2
N-MeHomoPhe-*N*-MePhe	40.2	3.86×10^{-5}	−4.4	0
N-Me2Nal-*N*-MePhe	38.9	3.57×10^{-5}	−4.4	7
N-MeCha-*N*-MePhe	34.6	2.79×10^{-5}	−4.5	17
N-MeOct-*N*-MePhe	32.3	2.45×10^{-5}	−4.6	26
N-Me1PyrenylAla-*N*-MePhe	0.5	0.02×10^{-5}	−6.6	83
Ac-*N*-MePhe-*N*-MePhe-$CONH_2$	6.8	0.33×10^{-5}	−5.5	29
propranolol	27.1	2.2×10^{-5}	−4.6	22
carbamazepine	26.4	1.9×10^{-5}	−4.7	19

Most of the DKPs are in the region where a positive in vivo CNS transport is predicted, except for 1PyrenylAla-*N*-MePhe and *N*-Me-1PyrenylAla-*N*-MePhe, as these interact too strongly with the phospholipids, which leads to retention in the membrane, as observed in the PAMPA assay. The evaluation of the mixture by IAMC is possible using HPLC-MS; however, it is not an easy task when one wishes to evaluate phospholipophilicity under physiological conditions, i.e., saline phosphate buffer (PBS) at pH 7.4.

The preparation and evaluation of these DKPs has helped to establish a number of rules about the passive transport across the BBB that will guide us in the design of a BBB-shuttle.

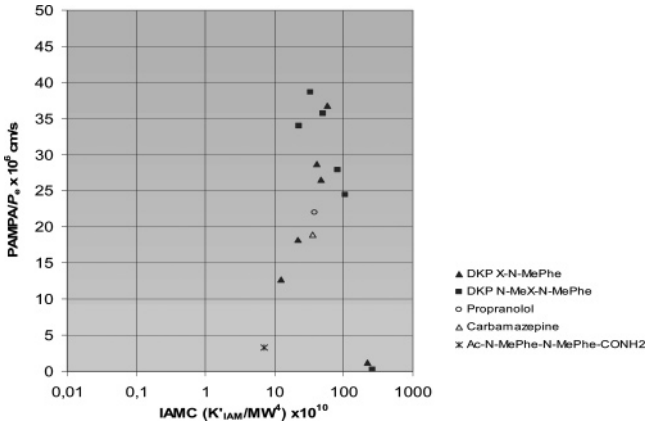
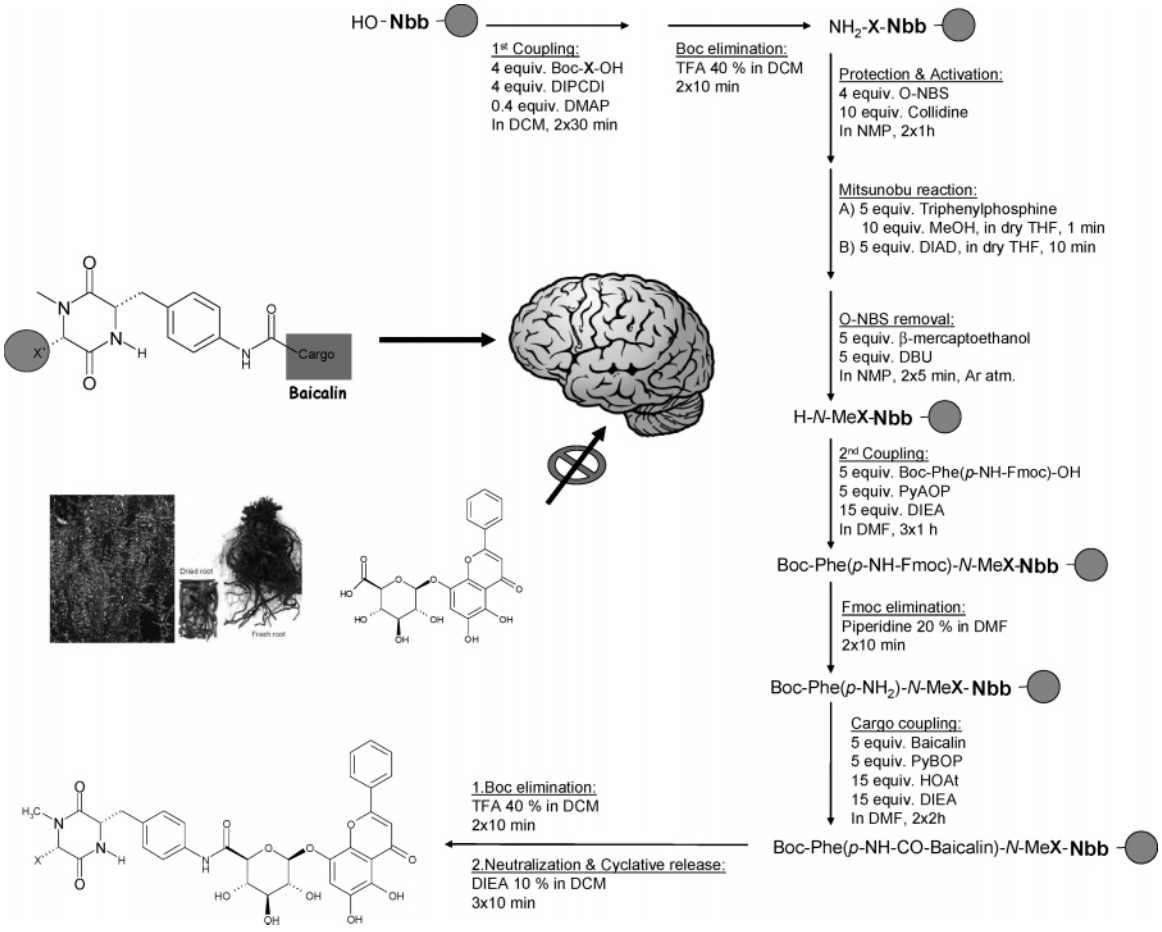


Figure 3. Comparison of the values measured by PAMPA (effective permeability, P_e) and by IAMC (capacity factor taking into account molecular weight, K'_{IAM}/MW^4) for the DKPs and control compounds in Table 1.

Scheme 3. Synthetic Methodology for the Preparation of DKP−Baicalin Constructs



DKPs as a BBB-Shuttle. Many active compounds cannot cross the BBB to reach their target sites inside the CNS. A BBB-shuttle based on a DKP or cyclic dipeptide scaffold could be used to send or shuttle anticancer drugs, antiretroviral agents, and other compounds that cannot cross the BBB, such as dopamine or MRI and PET diagnostic agents, thereby avoiding the undesired side effects of current therapy and providing a simpler and noninvasive technique than those currently used.

The BBB-shuttle cyclic dipeptide is a nonviral agent that could represent a new solution to bypass the problem of bioavailability of certain types of potential CNS therapeutic drugs.

The BBB-shuttle could be useful in several therapeutic fields. For example, the HIV virus can cross the BBB either during primary infection or at a later stage.[42−44] The brain might therefore serve as an hidden reservoir for HIV viral replication.[45,46] HIV in the brain and in the cerebrospinal fluid may be particularly resistant to therapy, because of the failure of antiretroviral drugs to penetrate the BBB.[47] The resulting infection leads to a number of CNS disorders, such as AIDS dementia complex and HIV encephalopathy.[48] Antiretroviral agents that cross the BBB or BBB-shuttles able to transport promising antivirals that cannot cross this barrier alone are urgently required.

Baicalin, a flavonoid isolated from the plant *Scutellaria baicalensis Georgi*, traditionally used in Chinese medicine, is an example of a compound with interesting properties. It is a non-nucleoside inhibitor of HIV-1 reverse transcriptase (NNRTI) and an inhibitor of the prolyl oligopeptidase associated with schizophrenia and bipolar affective disorder, but it cannot cross the BBB.[49−51]

Another field of interest of a BBB-shuttle is brain tumor cancer treatment, as many chemotherapy drugs are not suitable for this therapy because they do not have the capacity to cross the BBB.[52] A BBB-shuttle would also be of interest in the field of psychiatric diseases, such as schizophrenia, in epilepsy, and in neurodegenerative disorders, such as Parkinson's disease. As a result of the lack of transport of dopamine across the BBB, L-dopa, a precursor of dopamine, has been used in the treatment of Parkinson's disease since the 1960s. However, severe side effects are frequently observed within a few years of L-dopa therapy.[53]

To date, the methods used to administer drugs to the brain, either for therapy or diagnosis, are sometimes invasive, such

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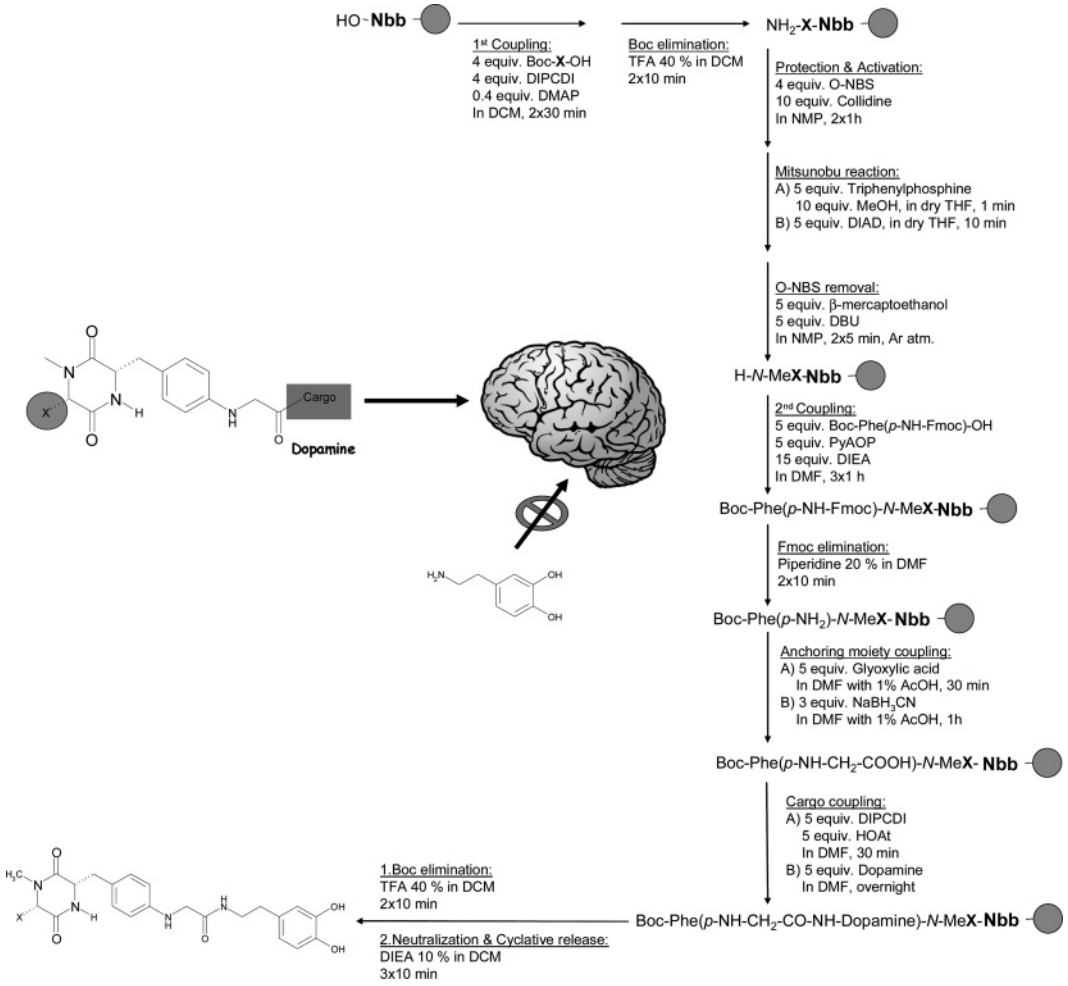
Scheme 4. Synthetic Methodology for the Preparation of DKP−Dopamine Constructs



as intracranial administration in the case of water-soluble anticancer drugs. In addition, temporary alteration of BBB integrity by osmotic disruption has been used to supplement chemotherapy for certain patients with brain tumors.[54] In other cases, drug administration to the brain has undesired side effects because of the high doses used to overcome the low permeability across this barrier. Sometimes the drug can be modified to improve its transport across the BBB,[55−61] by reducing drug size, increasing drug lipophilicity, etc. Administration of the drug to the brain by conjugation to a biological carrier[62] and intranasal delivery[63] are currently being explored to tackle BBB permeability to drugs.

The use of a BBB-shuttle approach implies that the search for novel drugs does not become limited only to compounds that have the capacity to cross the BBB. We selected the DKPs that showed the greatest capacity to cross the BBB as BBB-shuttles. These DKPs were modified with adequate functional groups for covalent fixation of the cargo. A DKP (BBB-shuttle) with the capacity to link a cargo with an amino or hydroxylic group and a DKP with the capacity to link a cargo with a carboxylic group were prepared. The cargo was linked to the BBB-shuttle such that, once inside the brain, it would not have to be detached from the shuttle to be active. Nonetheless, for future applications, the chemistry of the linkage could be optimized to enable facile hydrolysis of this bond in the appropriate location.[64]

Baicalin and dopamine were chosen as cargo. Baicalin was linked through the carboxylic group present in its sugar moiety, which is not needed for activity.[65] Dopamine was kept with its hydroxylic groups unprotected and was linked through its amino group.

The DKP−cargo constructs were prepared for the best side chains, Phe, HomoPhe, Cha, Oct, 2Nal, 1PyrenylAla, following the solid-phase Scheme 3 (for baicalin) and Scheme 4 (for dopamine). The DKP−baicalin and DKP−dopamine constructs were synthesized, purified, and characterized by MALDI-TOF, HPLC-MS, and HPLC and then evaluated by the PAMPA assay (Tables 2 and 3). *Scutellariae radix* extract and isolated baicalin

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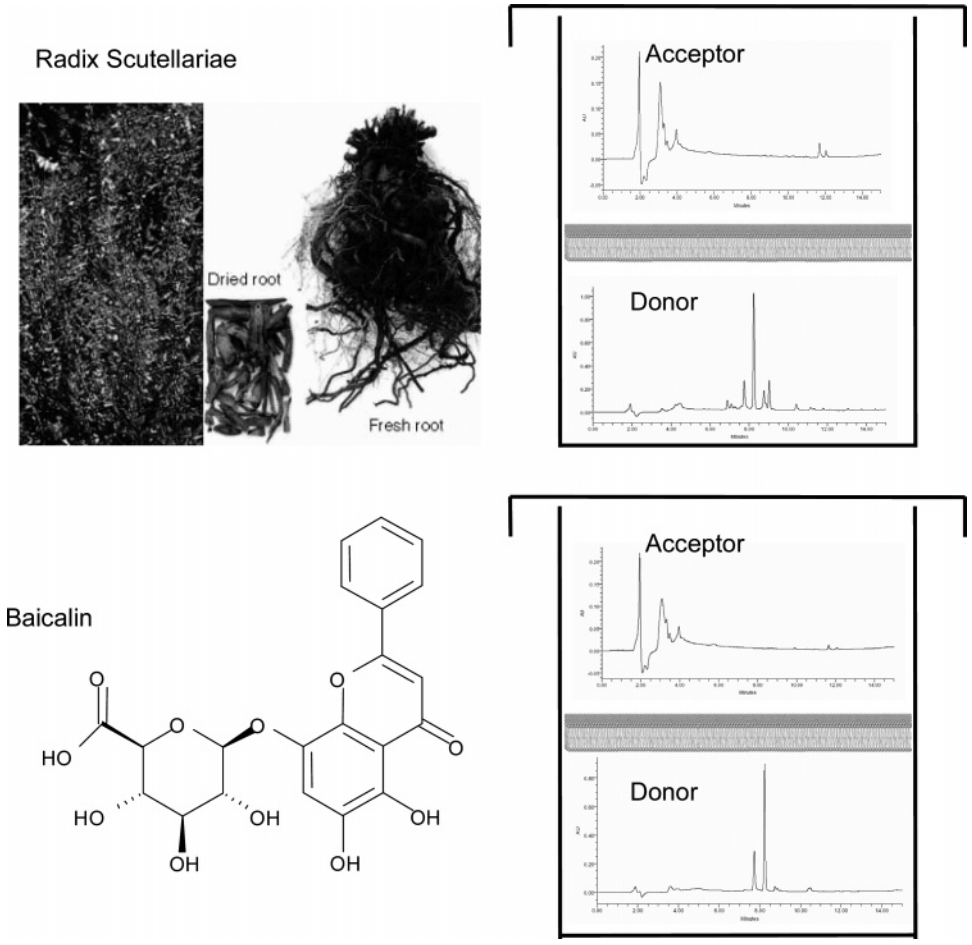

Figure 4. HPLC chromatograms of donor and acceptor PAMPA compartments at 4 h for the *S. radix* extract and the isolated baicalin, in a linear gradient from 0 to 100% MeCN in 15 min using a Symmetry C_{18} column (150 × 4.6 mm × 5 μm, 100 Å, Waters).

Table 2. Percentage of Transport after 4 h and Effective Permeability (P_e and log P_e) Found in the PAMPA for DKP−Baicalin Constructs and Baicalin (Cargo)

compound	% transport at 4 h	P_e (cm/ s)	log P_e
baicalin	—[a]	—[a]	—[a]
DKP Phe(*p*-NH-CO-baicalin)-*N*-MePhe	0.03	1.33×10^{-8}	−7.7
DKP Phe(*p*-NH-CO-baicalin)-*N*-MeHomoPhe	—[a]	—[a]	—[a]
DKP Phe(*p*-NH-CO-baicalin)-*N*-MeCha	0.76	3.65×10^{-7}	−6.4
DKP Phe(*p*-NH-CO-baicalin)-*N*-Me2Nal	7.22	3.70×10^{-6}	−5.4
DKP Phe(*p*-NH-CO-baicalin)-*N*-MeOct	1.04	4.97×10^{-7}	−6.3
DKP Phe(*p*-NH-CO-baicalin)-*N*-Me-1PyrenylAla	0.01	0.19×10^{-8}	−8.7

[a] These compounds were not detected in the acceptor well of the PAMPA after 4 h.

were also evaluated by the PAMPA assay (Figure 4). *S. radix* extract and isolated baicalin did not cross the PAMPA assay, but the BBB-shuttle constructs based on the DKP did carry baicalin through this assay (Table 2). The transport of the compounds was measured by HPLC of the acceptor compartments and confirmed by HPLC−MS and/or MALDI-TOF spectrometry, which also showed that there was no loss in the integrity of the DKP−cargo constructs during the PAMPA assay.

Although our DKP−cargo constructs enabled only limited transport of dopamine and baicalin, they nevertheless greatly increased the transport of these challenging compounds across the artificial model BBB membrane. The constructs based on *N*-MeOct and *N*-Me2Nal have been shown to be the most promising ones for the transport of dopamine and baicalin. However, the optimal BBB-shuttle may vary with the cargo.

In both cases, the linkage between the BBB-shuttle and the cargo stayed intact during diffusion through the PAMPA model. Moreover, the POP inhibition activity of the DKP Phe(*p*-NH−CO-baicalin)-*N*-MePhe was checked (48% at 100 μM) and was found to be as good as that of free baicalin (51% at 100 μM).

We also established methodology for the preparation of DKP-shuttles for other cargo, such as anticancer drugs, nanoparticles, and MRI and PET diagnostic agents (See the Supporting Information).

Experimental Section

Materials and Methods. Protected amino acids, handles, and resins were supplied by Luxembourg Industries (Tel-Aviv, Israel), Neosystem (Strasbourg, France), Calbiochem-Novabiochem AG (Laüfelfingen, Switzerland), Bachem AG (Bubendorf, Switzerland), or Iris Biotech (Marktredwitz, Germany). PyBOP was supplied by Calbiochem-Novabiochem AG. DIEA, DEA, DIPCDI, DMAP, KCN, $KHSO_4$, ninhydrin, and β-mercaptoethanol were obtained from Fluka Chemika (Buchs, Switzerland). HOBt was purchased from Albatros Chem. Inc. (Montreal, Canada). PyAOP was supplied by Applied Biosystems and HOAt from GL Biochem Shanghai Ltd (Shanghai, China). Solvents for peptide synthesis and RP-HPLC were obtained from Scharlau or SDS (Barcelona, Spain). Trifluoroacetic acid was supplied by Kali-

Table 3. Percentage of Transport after 4 h and Effective Permeability (P_e and log P_e) in the PAMPA for DKP−Dopamine Constructs and Also Dopamine (Cargo) and L-Dopa (Current Drug Used in the Market)

compound	% transport at 4 h	P_e (cm/ s)	log P_e
dopamine	—[a]	—[a]	—[a]
L-dopa	—[a]	—[a]	—[a]
DKP Phe(*p*-NH-CH_2-CO-NH-dopamine)-*N*-MePhe	0.08	3.61×10^{-8}	−7.4
DKP Phe(*p*-NH-CH_2-CO-NH-dopamine)-*N*-MeHomoPhe	0.02	0.76×10^{-8}	−8.1
DKP Phe(*p*-NH-CH_2-CO-NH-dopamine)-*N*-MeCha	0.05	2.28×10^{-8}	−7.6
DKP Phe(*p*-NH-CH_2-CO-NH-dopamine)-*N*-Me2Nal	0.24	1.15×10^{-7}	−6.9
DKP Phe(*p*-NH-CH_2-CO-NH-dopamine)-*N*-MeOct	0.67	3.21×10^{-7}	−6.5
DKP Phe(*p*-NH-CH_2-CO-NH-dopamine)-*N*-Me-1PyrenylAla	0.04	1.90×10^{-8}	−7.7

[a] These compounds were not be detected in the acceptor well of the PAMPA after 4 h.

Chemie (Bad Wimpfen, Germany). Other chemicals used were purchased from Aldrich (Milwaukee, WI) and were of the highest purity commercially available. All commercial reagents and solvents were used as received, with the exception of DCM and DMF. DMF was bubbled with nitrogen to remove volatile contaminants and stored over activated 4 Å molecular sieves (Merck, Darmstad, Germany). DCM was passed through a short column of Al_2O_3 (in the case of DCM used for peptide synthesis). PAMPA plates and PAMPA system solution were from pION (Woburn, MA USA). Porcine polar brain lipid extract (PBLEP) was purchased from Avantis Polar Lipids (Alabaster, AL). IAM column (10 × 4.6 mm, 12 μm, 300 Å, IAM.PC.DD2 column) was from Regis Technologies Inc. (Morton Grove, IL). Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (PE Biosystems, Foster City, CA), using an ACH matrix. HPLC chromatograms were recorded on a Waters model Alliance 2695 with a photodiode array detector 996 from Waters (Waters, Milford, MA) using a Symmetry C_{18} column (150 × 4.6 mm × 5 μm, 100 Å, Waters), H_2O (0.045% TFA) and MeCN (0.036% TFA) as solvents at 1 mL/min, and *Millenium* version 4.0 software. HPLC−MS [Waters model Alliance 2796, quaternary pump, UV/vis dual λ absorbance detector Waters 2487, ESI-MS model Micromass ZQ and *Masslynx* version 4.0 software (Waters)] was done using a Symmetry 300 C_{18} column (150 × 3.9 mm × 5 μm, 300 Å, Waters) with H_2O (0.1% formic acid) and MeCN (0.07% formic acid) as solvents at 1 mL/min. The products were purified in a Waters 600 with a dual λ absorbance detector (Waters 2487, Waters) and a Symmetry C_{18} column (100 × 30 mm × 5 μm, 100 Å, Waters) with H_2O (0.1% TFA) and MeCN (0.05% TFA) as solvents at 10 mL/min. ^{1}H NMR spectra were obtained at 25 °C with a Varian Mercury 400 NMR spectrometer using CD_3OD as a solvent.

ZGP-AMC was obtained from Bachem (Bubendorf, Switzerland). POP was obtained by expression in *Escherichia coli* and subsequent affinity purification using an His tail fusion as reported previously.[51] Fluorescence was measured using a Bio-Tek FL600 fluorescence plate reader (Bio-Tek Instruments, Winooski, VT).

Synthesis of Nbb Handle. 4-Bromomethyl-3-nitrobenzoic acid (4.5 g, 17.3 mmols) was placed in a round-bottom flask provided with reflux system, and an aqueous $NaHCO_3$ saturated solution was added (135 mL). The reaction was stirred and allowed to stand at 90−95 °C. The progress of the reaction was monitored by TLC (chloroform/MeOH/AcOEt, 100:50:0.1, v/v) or by HPLC of an acidified aliquot in a gradient from 0 to 100% of MeCN in 15 min in a Symmetry C_{18} column (150 × 4.6 mm × 5 μm, 100 Å, Waters). Retention time for the final product was 7.1 min; retention time for the initial product was 10.4 min. The reaction was completed in 30 min. The solution obtained was hot-filtered, the reaction was quenched with HCl (12N) to pH 1−2, the crude product was extracted with AcOEt (3 × 100 mL), and the organic fractions were combined, washed with a saturated NaCl aqueous solution, and dried over anhydrous $MgSO_4$. The AcOEt was evaporated in vacuo. The product was obtained as a pale yellow solid. Yield: 80%, 2.73 g. HPLC t_R: 7.12 min (linear gradient 0−100% MeCN in 15 min). HPLC-MS: 196 Da. TLC: $R_f = 0.3$ (chloroform/MeOH/AcOEt, 100:50:0.1, v/v). ^{1}H NMR (CD_3OD, 400 MHz, δ ppm): 4.98 (s, 2H), 7.96 (d, $J = 21$, 1H), 8.26 (dd, $J = 4$, $J = 20$, 1H), 1.56−1.24 (m, 12H), 8.56 (d, $J = 4$, 1H).

General Protocols for Solid-Phase Synthesis. Syntheses were performed on a 100 μmol scale/each, in all cases L-amino acids were used. Solid-phase peptide elongation and other solid-phase manipulations were done manually in polypropylene syringes, each fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Washings between synthetic steps were done with DMF (5 × 0.5 min) and DCM (5 × 0.5 min) using 5 mL of solvent/g of resin each time. During couplings the mixture was allowed to react with intermittent manual stirring.

Identification Tests. The Kaiser colorimetric assay was used for the detection of solid-phase-bound primary amines,[66] while the De Clercq test was used for secondary amines bound to solid-phase.[67]

Initial Conditioning of Resin. The *p*-MBHA resin was conditioned by washing with DCM (5 × 30 s) followed by a 40% TFA solution in DCM (1 × 30 s and 2 × 5 min). This acid treatment was followed by a neutralization step with DIEA 5% in DCM (3 × 2 min) and finally the resin was washed with DCM (5 × 30 s).

Coupling of Nbb Handle to *p*-MBHA Resin. Nbb handle (97 mg, 5 equiv) in DCM (3 mL/g of resin) and DIPCDI (77 μL, 5 equiv) were sequentially added to the *p*-MBHA resin (164 mg). The mixture was left to stir overnight. The solvent was then removed by suction, the resin was thoroughly washed, and the reaction was monitored with the Kaiser test.

Boc Group Removal. The Boc group was removed by treating the resin with 40% (v/v) TFA in DCM (3−4 mL/g of resin, 2 × 10 min). In solution, the Boc group was removed using 50% TFA in DCM for 1 h. DCM was then evaporated under reduced pressure.

Fmoc Group Removal. The Fmoc group was removed by treating the resin with 20% piperidine in DMF (3−4 mL/g of resin, 2 × 1 min and 1 × 20 min). To remove the Fmoc group in solution, the compound was dissolved in DMF (100 mg/mL) and the solution was kept at 0 °C; DEA was then added to reach 5%. The reaction was left to reach room temperature and then left to stand at room temperature for 3 h under stirring. To remove the base and the remaining Fmoc byproduct, the compound was purified.

Coupling Methods: **Method 1, Coupling of the First Amino Acid onto the Nbb Handle**. Amino acid derivative (4 equiv), DIPCDI (62 μL, 4 equiv), and DMAP (5 mg, 0.4 equiv) in DCM (1−3 mL/g of resin) were sequentially added to the resin. The mixture was allowed to react with intermittent manual stirring for 30 min. The solvent was then removed by suction, the resin was thoroughly washed, and the coupling was repeated once.

Method 2, Coupling of the Second Amino Acid. Protected amino acid (5 equiv) and PyAOP (260 mg, 5 equiv) in DMF (1−3 mL/g of

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resin) were sequentially added to the resin followed with DIEA (255 μL, 15 equiv). The mixture was left to react with intermittent manual stirring for 1 h. The solvent was removed by filtration, the resin was washed as indicated above, and the coupling was repeated two more times. The extent of the coupling was checked by the De Clercq test.

Amino Acid *N*-Alkylation. The *N*-methylation of the amino acid derivatives was performed using the methods described by Biron et al.[35] and Yang et al.[36] This process can be divided in to three steps: (A) protection and activation with O−NBS, (B) Mitsunobu reaction, and (C) O−NBS removal.

(A) Protection and Activation with O−NBS. To perform the protection, O−NBS (88 mg, 4 equiv) and collidine (170 μL, 10 equiv) in NMP were added to the resin. The reaction was left with intermittent manual stirring for 1 h and this step was repeated once and checked by the Kaiser test.

(B) Mitsunobu Reaction. The Mitsunobu reagents, triphenylphosphine (133 mg, 5 equiv), and MeOH (24 μL, 10 equiv) in dry THF were added to the resin and left for 1 min; afterward, without filtering, DIAD (98 μL, 5 equiv) was added in dry THF and left for another 10 min.

(C) O−NBS Removal. To proceed to O−NBS removal, β-mercaptoethanol (70 μL, 10 equiv) and DBU (75 μL, 5 equiv) in NMP were added to the resin, and the mixture was left to react for 5 min under an argon atmosphere. This process was repeated once.

Introduction of the Anchoring Moiety by Reductive Amination. The anchoring moiety was introduced by adding glyoxylic acid (37 mg, 5 equiv) in DMF with 1% AcOH to the resin and leaving it for 30 min. The solvent and reagents were removed by filtration, the resin was washed, and then $NaBH_3CN$ (19 mg, 3 equiv) in DMF with 1% AcOH was added to perform the reduction for 1 h.

Coupling of the Cargo. Depending on the nature of the cargo, it was linked to the BBB-shuttle through several types of chemical bonds.

For Cargo with a Carboxylic Group (e.g., Baicalin). The corresponding BBB-shuttle provided with a NH_2 group was used and was reacted with cargo−COOH (224 mg of baicalin, 5 equiv), using PyBOP (260 mg, 5 equiv) and HOAt (196 mg, 15 equiv) as coupling reagents and DIEA (256 μL, 15 equiv) as a base in DMF for 2 h.

For Cargo with an Amine or Alcohol Group (e.g., Dopamine). This reaction was done in two steps. First, the carboxylic group was activated with DIPCDI (78 μL, 5 equiv) and HOAt (68 mg, 5 equiv) in DMF for 30 min, after which the solvent was removed by suction and the resin was washed. The coupling was performed using NH_2-cargo (98 mg dopamine, 5 equiv) in DMF. The mixture was left to react with intermittent manual stirring overnight. The solvent was removed by filtration and the resin was washed.

Neutralization−Cyclization and Cleavage All in One Step. Only by treating the resin with 10% DIEA in DCM (3 × 10 min) was neutralization−cyclization and cleavage accomplished. The filtrates were collected, DCM was evaporated under N_2, and the residue was solved in H_2O:MeCN (1:1) and lyophilized.

Product Workup and RP-HPLC Purification. The mono and di-*N*-methylated DKPs of the two libraries which did not need to be purified by RP-HPLC were directly desalted using a DOWEX MR-3 mixed bed resin overnight. The remaining compounds were purified by reverse-phase HPLC using a Symmetry C_{18} column (100 × 30 mm × 5 μm, 100 Å, Waters), at 10 mL/min flow with the following solvents: A, H_2O with 0.1% TFA; B, MeCN with 0.05% TFA. The DKP−dopamine and DKP−baicalin constructs, as well as the DKP Phe(*p*-NH_2)-*N*-MePhe, *N*-MePhe-*N*-MePhe(*p*-NH_2), and Phe(*p*-NH−CH_2−COOH)-*N*-MePhe, were directly purified by RP-HPLC.

Product Characterization. The identity of the compounds synthesized was confirmed using MALDI-TOF mass spectrometry or HPLC-MS. Purity was checked by reverse phase HPLC.

Characterization of the First Library of DKPs. Reverse phase HPLC: linear gradient from 0 to 100% MeCN in 15 min using a Symmetry C_{18} column (150 × 4.6 mm × 5 μm, 100 Å, Waters). t_R: Phe-*N*-MePhe, 9.7 min; HomoPhe-*N*-MePhe, 10.3 min; Cha-*N*-MePhe, 11.1 min; 2Nal-*N*-MePhe, 11.3 min; Oct-*N*-MePhe, 11.4 min; 1PyrenylAla-*N*-MePhe, 13.5 min; PyrAla-*N*-MePhe, 5.9 min; Gly-*N*-MePhe, 6.7 min; Pro-*N*-MePhe, 7.8 min; Phg-*N*-MePhe, 8.8 and 9.4 min; *N*-MePhe-*N*-MePhe, 10.6 min; Pro(Phe)-*N*-MePhe, 10.8 min; Tic-*N*-MePhe, 10.4 min; Tyr-*N*-MePhe, 8.3 min; *N*-MeTrp restricted-*N*-MePhe, 10.6 min. Mass spectrometry (MALDI-TOF): Phe-*N*-MePhe, 309.0 Da; HomoPhe-*N*-MePhe, 325.2 Da; Cha-*N*-MePhe, 315.2 Da; 2Nal-*N*-MePhe, 359.7 Da; Oct-*N*-MePhe, 303.2 Da; 1PyrenylAla-*N*-MePhe, 433.2 Da; PyrAla-*N*-MePhe, 310.1 Da; Gly-*N*-MePhe, 219.6 Da; Pro-*N*-MePhe, 258.5 Da; Phg-*N*-MePhe, 295.6 Da; *N*-MePhe-*N*-MePhe, 323.2 Da; Pro(Phe)-*N*-MePhe, 335.1 Da; Tic-*N*-MePhe, 321.7 Da; Tyr-*N*-MePhe, 325.7 Da; *N*-MeTrp restricted-*N*-MePhe, 358.5 Da. Yield: Phe-*N*-MePhe, 5.2%; HomoPhe-*N*-MePhe, 24%; Cha-*N*-MePhe, 5.1%; 2Nal-*N*-MePhe, 4.5%; Oct-*N*-MePhe, 9.9%; 1PyrenylAla-*N*-MePhe, 4.2% (after RP-HPLC purification), PyrAla-*N*-MePhe, 7.8%; Gly-*N*-MePhe, 8.3%; Pro-*N*-MePhe, 15%; Phg-*N*-MePhe, 33%; *N*-MePhe-*N*-MePhe, 33%; Pro(Phe)-*N*-MePhe, 19%; Tic-*N*-MePhe, 5.6%; Tyr-*N*-MePhe, 41%; *N*-MeTrp restricted-*N*-MePhe, 7.6%.

Characterization of the Second Library of DKPs. Reverse phase HPLC: linear gradient from 0 to 100% MeCN in 15 min using a Symmetry C_{18} column (150 × 4.6 mm × 5 μm, 100 Å, Waters). t_R: *N*-MePhe-*N*-MePhe, 10.4 min; *N*-MePhe-*N*-MeHomoPhe, 11.2 min; *N*-MePhe-*N*-Me2Nal, 11.9 min; *N*-MePhe-*N*-MeOct, 12.4 min; *N*-MePhe-*N*-MeCha, 12.5 min; *N*-MePhe-*N*-Me-1PyrenylAla, 14.0 min. Mass spectrometry (MALDI-TOF): *N*-MePhe-*N*-MePhe, 323.2 Da; *N*-MePhe-*N*-MeHomoPhe, 337.1 Da; *N*-MePhe-*N*-Me2Nal, 373.2 Da; *N*-MePhe-*N*-MeOct, 317.2 Da; *N*-MePhe-*N*-MeCha, 329.2 Da; *N*-MePhe-*N*-Me-1PyrenylAla, 447.2 Da. Yield: *N*-MePhe-*N*-MePhe, 50%; *N*-MePhe-*N*-MeHomoPhe, 75%; *N*-MePhe-*N*-Me2Nal, 61%; *N*-MePhe-*N*-MeOct, 90%; *N*-MePhe-*N*-MeCha, 95%; *N*-MePhe-*N*-Me-1PyrenylAla, 60%.

Characterization of the DKP Phe(*p*-NH−CO-cargo)-*N*-MePhe and Its Analogous DKP Phe(*p*-NH−CO-cargo)-*N*-MeX, Where X = HomoPhe, Cha, 2Nal, 1PyrenylAla, Oct and Cargo = Baicalin. Reverse phase HPLC: linear gradient from 0 to 100% MeCN in 15 min using a Symmetry C_{18} column (150 × 4.6 mm × 5 μm, 100 Å, Waters). t_R: Phe(*p*-NH−CO-baicalin)-*N*-MePhe, 9.3 min; Phe(*p*-NH−CO-baicalin)-*N*-MeHomoPhe, 8.9 min; Phe(*p*-NH−CO-baicalin)-*N*-MeCha, 8.6 min; Phe(*p*-NH−CO-baicalin)-*N*-Me2Nal, 9.5 min; Phe(*p*-NH−CO-baicalin)-*N*-Me-1PyrenylAla, 9.5 min; Phe(*p*-NH−CO-baicalin)-*N*-MeOct, 10.5 min (confirmed by HPLC−MS). Mass spectrometry (MALDI-TOF): Phe(*p*-NH−CO-baicalin)-*N*-MePhe, 752.2 Da; Phe(*p*-NH−CO-baicalin)-*N*-MeHomoPhe, 766.1 Da; Phe(*p*-NH−CO-baicalin)-*N*-MeCha, 758.1 Da; Phe(*p*-NH−CO-baicalin)-*N*-Me2Nal, 801.4 Da; Phe(*p*-NH−CO-baicalin)-*N*-Me-1PyrenylAla, 876.3 Da; Phe(*p*-NH−CO-baicalin)-*N*-MeOct, 745.4 Da. Total yields after synthesis and RP-HPLC purification: Phe(*p*-NH−CO-baicalin)-*N*-MePhe, 1.2%; Phe(*p*-NH−CO-baicalin)-*N*-MeHomoPhe, 1.7%; Phe(*p*-NH−CO-baicalin)-*N*-MeCha, 2.4%; Phe(*p*-NH−CO-baicalin)-*N*-Me2Nal, 2.5%; Phe(*p*-NH−CO-baicalin)-*N*-Me-1PyrenylAla, 1.4%; Phe(*p*-NH−CO-baicalin)-*N*-MeOct, 1.1%.

Characterization of the DKP Phe(*p*-NH−CH_2−CO−NH-cargo)-*N*-MePhe and Its Analogous DKP Phe(*p*-NH−CH_2−CO−NH-cargo)-*N*-MeX, Where X = HomoPhe, Cha, 2Nal, 1PyrenylAla, Oct and Cargo = Dopamine. Reverse phase HPLC: linear gradient from 15 to 65% MeCN in 15 min using a Symmetry C_{18} column (150 × 4.6 mm × 5 μm, 100 Å, Waters). t_R: Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-MePhe, 7.8 min; Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-MeHomoPhe, 7.8 min; Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-MeCha, 9.4 min; Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-Me2Nal, 9.6 min; Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-Me-1PyrenylAla, 11.7 min; Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-MeOct, 10.6 min (confirmed by HPLC-MS). Mass spectrometry (HPLC-MS): Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-MePhe, 516.5 Da; Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-MeHomoPhe, 530.3 Da; Phe(*p*-NH−CH_2−

CO−NH-dopamine)-*N*-MeCha, 522.5 Da; Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-Me2Nal, 566.2 Da; Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-Me-1PyrenylAla, 640.4 Da; Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-MeOct, 510.4 Da. Total yields after synthesis and RP-HPLC purification: Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-MePhe, 10.8%; Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-MeHomoPhe, 9.1%; Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-MeCha, 7.2%; Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-Me2Nal, 10.9%; Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-Me-1PyrenylAla, 9.6%; Phe(*p*-NH−CH_2−CO−NH-dopamine)-*N*-MeOct, 8.2%.

Synthesis and Characterization of the Control Linear Dipeptide Ac-*N*-MePhe-*N*-MePhe-$CONH_2$. The linear dipeptide was synthesized on a 50 μmol scale using Sieber resin. For the coupling of the first amino acid, Fmoc-*N*-MePhe (81 mg, 4 equiv), PyBOP (104 mg, 4 equiv) and HOAt (79 mg, 12 equiv) in DMF (1−3 mL/g of resin), and DIEA (103 μL, 12 equiv) were sequentially added to the resin. The mixture was left to react with intermittent manual stirring for 90 min. The solvent was removed by filtration, the resin was washed, and the coupling was repeated once. The extent of the coupling was checked by the Kaiser test.

For the second amino acid, Fmoc-Phe (78 mg, 4 equiv), PyBOP (104 mg, 4 equiv) and HOAt (79 mg, 12 equiv) in DMF (1−3 mL/g of resin), and DIEA (103 μL, 12 eq) were sequentially added to the resin. The mixture was left to react with intermittent manual stirring for 90 min. The solvent was removed by filtration, the resin was washed, and the coupling was repeated two more times. The extent of the coupling was checked by the De Clercq test.

N-methylation of the second amino acid was performed on the solid-phase. The *N*-terminal acetylation was done using Ac_2O (236 μL, 50 equiv) and DIEA (428 μL, 50 equiv) in DCM (1−3 mL/g of resin). The mixture was left to react with intermittent manual stirring for 20 min. The solvent was removed by filtration, and the resin was washed. The cleavage was performed using with 2% (v/v) TFA in DCM (10 mL/g of resin, 4 × 3 min). The filtrates were collected, and the DCM was evaporated under N_2. The residue was dissolved in H_2O:MeCN (1:1) and then lyophilized. Reverse phase HPLC: linear gradient from 0 to 100% MeCN in 15 min using a Symmetry C_{18} column (150 × 4.6 mm × 5 μm, 100 Å, Waters). t_R: Ac-*N*-MePhe-*N*-MePhe-$CONH_2$, 8.2 min (confirmed by HPLC-MS). Mass spectrometry (MALDI-TOF): Ac-*N*-MePhe-*N*-MePhe-$CONH_2$, 381.2 Da. Total yield after synthesis and RP-HPLC purification: Ac-*N*-MePhe-*N*-MePhe-$CONH_2$, 41%.

Parallel Artificial Membrane Permeability Assay. The PAMPA assay was used to determine the capacity of compounds to cross the BBB by passive diffusion.[68,69] The effective permeability of the compounds was measured in triplicate at an initial concentration of 200 μM. The buffer solution was prepared from a concentrated one, commercialized by pION, following the manufacturer's instructions. pH was adjusted to 7.4 using a 0.5M NaOH solution. The compound of interest was dissolved to the desired concentration (200 μM). The PAMPA sandwich was separated and the donor well was filled with 200 μL of the compound solution to be studied. The acceptor plate was placed into the donor plate, ensuring that the underside of the membrane was in contact with buffer. Then 4 μL of the mixture of phospholipids (20 mg/mL) in dodecane was added to the filter of each well and 200 μL of buffer solution was added to the each acceptor well. The plate was covered and incubated at room temperature in a saturated humidity atmosphere for 4 h under orbital agitation at 100 rpm. After the 4 h, 150 μL/well from the donor plate and 150 μL/well from the acceptor plate were transferred to HPLC vials, and 100 μL/each sample was injected in a HPLC reverse phase Symmetry C_{18} column (150 × 4.6 mm × 5 μm, 100 Å, Waters). The transport was also confirmed by MALDI-TOF spectrometry and/or HPLC-MS of aliquots of the acceptor wells.

The phospholipid mixture used was a porcine polar brain lipid extract. Composition: 12.6% phosphatidylcholine (PC), 33.1% phosphatidylethanolamine (PE), 18.5% phosphatidylserine (PS), 4.1% phosphatidylinositol (PI), 0.8% phosphatidic acid, and 30.9% of other compounds. The percentage of transport after 4 h was calculated as was the effective permeability, using eq 1

$$P_e = \frac{-218.3}{t}\log\left[1 - \frac{2C_A(t)}{C_D(t_0)}\right] \times 10^{-6}\ \text{cm/s} \qquad (1)$$

where t is time (h); $C_A(t)$ is the compound concentration at the acceptor well at time t, and $C_D(t_0)$ is the compound concentration at the donor well at 0 h.

Immobilized Artificial Membrane Chromatography. Retention times were determined using an IAMC column with phosphatidylcholine (PC), the major phospholipid in cell membranes, which was covalently immobilized (10 × 4.6 mm, 12 μm, 300 Å, IAM.PC.DD2 column, Regis Technologies Inc.).

The compounds were detected by UV absorption at 220 nm. The chromatograms were obtained using an HPLC working isocratically with a mobile phase containing 10 mM phosphate buffer, 2.7 mM KCl, and 137 mM NaCl at pH 7.4 and 20% (v/v) MeCN. The retention times (t_R) were transformed into capacity factors (K'_{IAM}) following eq 2

$$K'_{IAM} = (t_R - t_0)/(t_0 - t_{connectors}) \qquad (2)$$

where t_R is the compound retention time (min); t_0 is the citric acid retention time (min), and $t_{connectors}$ is the citric acid retention time without column (min).

POP Activity Assay. POP activity was determined as follows: For each reaction, activity buffer (131 μL, 100 mM of Na/K phosphate buffer, pH 8.0) was preincubated for 15 min at 37 °C with POP (7 nM) and the corresponding inhibitor solution (3 μL). A 5.3 mM stock solution of inhibitor was prepared in H_2O:MeCN (1:1, v/v). After preincubation, ZGP-AMC (10 μL, 3 mM in 40% of 1,4-dioxane) was added, and the reaction was incubated for 1 h at 37 °C. The reaction was stopped with sodium acetate (150 μL, 1M, pH 4), at which point the formation of AMC was measured fluorimetrically. The excitation and emission wavelengths were 360/40 and 485/20 nm, respectively. A control with the same amount of MeCN was performed.

Conclusions

In this paper, we have introduced, for the first time, the concept of using DKPs as shuttles to the brain for the delivery of drugs that cross the BBB with difficulty. This new approach is illustrated with the cases of dopamine and baicalin, and for both compounds, our data using the PAMPA assay demonstrates that the new prodrugs are able to cross a model of the BBB membrane. Our results are only preliminary and cell-based in vitro assays and in vivo studies will follow as well as the application of the DKPs as BBB-shuttles for other potential CNS drugs and diagnostic agents.

From the synthetic point of view, the all-in-solid-phase methods described are highly convenient, as they allow the effective synthesis of two libraries of mono- and di-*N*-methylated DKPs, as well as the DKP−dopamine and DKP−baicalin constructs. A comparison of the transport properties of all the DKPs synthesized highlights the importance of the capacity to form hydrogen bonds for the optimization of permeability across the BBB. It is important to take into account that the simple rule "*the smaller the number of potential hydrogen bonds that*

(68) Balimane, P. V.; Pace, E.; Chong, S.; Zhu, M.; Jemal, M.; Van Pelt, C. K. *J. Pharm. Biomed. Anal.* **2005**, *39*, 8−16.
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can be formed, the better the transport" is inappropriate, as it can lead to compounds that are too hydrophobic and consequently retained in the lipid membrane. In addition, from the methodological point of view, we have demonstrated the usefulness of performing PAMPA assays for the evaluation of mixtures. In our format, both the throughput and the reliability of the method are improved.

Our BBB-shuttle cyclic dipeptide may provide a new solution to bypass the problem of bioavailability of certain types of potential CNS therapeutic drugs. This approach could be useful to transport promising antiretroviral agents and chemotherapy drugs across the BBB and into the brain, thereby reducing the severe side effects caused by the high doses currently used to overcome low permeability. Our BBB-shuttle approach implies that the search for novel drugs does not become limited only to compounds that have the capacity to cross the BBB.

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Supporting Information Available: Methodology for the preparation of DKP-shuttles for other cargo, including characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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