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Solution-Phase Combinatorial Synthesis and Evaluation of Piperazine-2,5-dione Derivatives

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Abstract—An efficient one-pot synthesis of a 61-membered combinatorial chemistry library of piperazine-2,5-diones was accomplished. Results of combinatorial synthesis, purification, analysis, and biological evaluation are described.

Introduction

Combinatorial chemistry[1–3] is providing a new and improved approach to the discovery of new and better pharmacological agents. Small molecule libraries have been rapidly accessed using a variety of methodologies and techniques that have been developed for use in solid-phase[4,5] as well as solution-phase synthesis.[6] Although solid-phase synthesis has been at the forefront of combinatorial chemistry, parallel solution-phase synthesis is an interesting alternative approach. The advantages that characterize solution-phase synthesis include validation time, facility of manipulations and the diversity of reactions that can be performed.

The synthesis of piperazine-2,5-dione molecules has received continuing interest because of their spectrum of pharmacological activities. Examples of biologically active 3,6-disubstituted piperazine-2,5-diones include antibiotics such as terezine A,[7] the safracins,[8] and albonoursin,[9] which also inhibits the growth of transplantable solid tumors in mice. Derivatives of piperazine-2,5-diones have shown potential as therapeutic agents and are used in medicine as antibiotics, synthetic vaccines, and in cancer therapy.[10] In this paper we wish to report the fast and flexible generation of an array of piperazine-2,5-dione derivatives for the rapid identification of potential cytotoxic agents.

Synthetic Chemistry

Methods for the synthesis of piperazine-2,5-diones include cyclisation of dipeptide derivatives, intramolecular attack of amines on activated carbonyl groups, cyclization of *N*-pyruvoyl amino acid amides, intramolecular Diels–Alder reaction, and synthesis from α-haloacyl derivatives of amino acid esters.[11] However, relatively few methods afford an approach amenable to combinatorial synthesis that is not reliant on amino acid building blocks. Therefore, we tested the suitability of condensation of aldehydes with 1,4-diacetylpiperazine-2,5-dione in the presence of base[12] to generate symmetrical and unsymmetrical derivatives in a one-pot parallel solution-phase combinatorial synthesis, paying particular attention to ease of isolation and purity of the target product. The approach was modified, as described below, for the construction of a solution combinatorial library.

Our approach to the design and synthesis of a piperazine-2,5-dione library consisted of the following steps: (i) Retain the core piperazine-2,5-dione ring, a structural element of several biologically active piperazine-2,5-dione natural products. (ii) Develop a practical synthetic procedure that allowed combinatorial synthesis. (iii) Have a good diversity in the library by choosing a variety of the aldehyde component such as aromatic, heteroaromatic, aliphatic, and α,β-unsaturated aldehydes. (iv) Include aldehyde components that contain polysubstituted aromatic functionality present in other bioactive natural products such as lamellarin G, trimethyl ether.[13] (v) Combine these elements to generate three combinatorial sets of compounds yielding, ideally, a small set of molecules sufficiently active to serve as leads in further investigations. (vi) Gain some preliminary biological data by testing members of the library, using the brine shrimp lethality assay as an antitumor prescreen.

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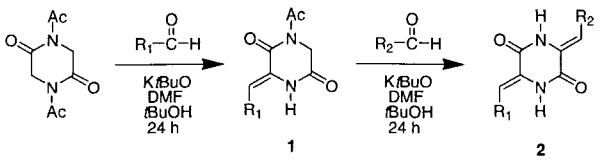
Commercial availability and appropriate functionalities were selection factors used to generate three sets of compounds consisting of (a) aliphatic, (b) mono-substituted aromatic, and (c) heteroaromatic and poly-substituted aromatic derivatives. The general procedure developed for the preparation of a piperazine-2,5-diones combinatorial library (Scheme 1) is now described. 1,4-Diacetylpiperazine2,5-dione (2 mmol) was placed in a library of quick-fit reacti-vials supported in an aluminium reacti-block at 25 °C. Dimethyl formamide (4 mL) and the first aldehyde (2 mmol) were added and the mixture stirred until a homogenous solution resulted. *tert*-Butyl alcohol (4 mL) and potassium *tert*-butoxide (2 mmol) were added and the reaction mixture stirred for 24 h at 25 °C. The second aldehyde (2 mmol) was added followed by further potassium *tert*-butoxide (2 mmol). The reaction mixture was stirred for another 24 h at 25 °C and then quenched with acetic acid (4 mL). It was essential to alter the parallel purification procedure on the basis of the components used in the synthesis of the sets of compounds. The conditions used for aromatic derivatives and aliphatic derivatives were first optimized on an individual target compound of the library. The following parallel purification procedures were developed. For aromatic derivatives, the reaction mixture was poured into a library of beakers, each containing water (20 mL). The product that precipitated was filtered off using a tandem filtration apparatus and the residual solid dried in vacuo, analyzed, and tested. For aliphatic derivatives, the reaction mixture was extracted in the quick-fit micro-vial library using ethyl acetate (3×10 mL). The combined organic layers were transferred to a library of beakers, dried ($MgSO_4$), filtered into another quick-fit micro-vial library using a tandem filtration apparatus, and the solvent removed in vacuo using a multi-line vacuum adaptor. The dried product was analyzed and tested.

The diversely substituted products (entries 1–70, Table 1)[14] obtained from the solution combinatorial synthesis were identified by a combination of HPLC/EI–MS and ^{1}H NMR techniques. Analysis of the samples using mass spectroscopy was an efficient approach, which provided unambiguous evidence that the combinatorial synthesis was very effective and indicated that all identified products were obtained in greater than 85% purity. All samples gave a mass spectral result that was assigned to either the expected product (87% of the library), or a mono or disubstituted by-product (10% of the library). Only two cases failed to react under the standard combinatorial reaction conditions (entries 13 and 18, Table 1). Isolation of mono or disubstituted by-products was a result of the nonreactivity of one of the aldehyde components under the standard combinatorial synthesis conditions. This was noteworthy when *para*-hydroxybenzaldehyde was used as an aldehyde component. The phenol hydroxyl group must be acting as a competing proton source and thus hindering the aldol condensation reaction that forms the substituted piperazine-2,5-dione products. Yields of products varied and were typically low in the aliphatic set of compounds and high in the aromatic sets of compounds. The combinatorial reaction conditions were not optimized for any one individual compound yet rapidly provided 87% of the expected di-substituted library formed under one standard set of combinatorial reaction conditions. The ease of scale of the solution combinatorial synthesis provided sufficient quantities of the piperazine-2,5-dione derivatives for biological testing.



Scheme 1.

Biological Evaluation

In order to identify potential lead structures for further cytotoxicity studies and identify the solubility of the piperazine-2,5-dione derivatives for further biological assays we carried out the brine shrimp assay for toxicity.[15] A positive correlation exists between brine shrimp toxicity and 9 KB cytotoxicity assay and the brine shrimp assay is accurate in predicting in vivo activity as cytotoxicity in a series of human solid-tumor cell lines ($p = 0.033$–0.334).[16] We validated our assay conditions using caffeine[15] and then tested the library of disubstituted piperazine-2,5-diones. *Artemia salina* eggs were kept for 24 h in artificial seawater as described previously,[15] and the nauplii ($n = \sim 20$) were brought into a 24-well micro-titre plate with brine (980 μL). The compounds to be tested were added in various concentrations (1–100 μg dissolved in 20 μL of DMSO). Each concentration was in quadruplicate. DMSO was required to improve compound solubility. The maximum concentration tested was 100 μg/mL due to the general insolubility of these compounds above this level. After incubation at 26 °C for 24 h, surviving larvae were counted and compared to the DMSO controls. The control deaths were <10%. The general levels of inhibition are reported in Table 1. Thirteen compounds (**2aa**, **2be**, **2de**, **2ee**, **2ef**, **2ms**, **2nn**, **2no**, **2ns**, **2ps**, **2qr**, **2qs**, and **2rr**) caused a marked inhibition of all nauplii movement at 100 μg/mL but did not cause nauplii death. LD_{50}s were determined for the three most potent compounds, **2np**, **2nq** and **2oq** and were calculated from the dose–response curves by probit analysis,[17] as being 60, 36, and 18 μg/mL, respectively.

From the general levels of inhibition and the LD_{50} values the mono-functionalised aromatic analogues (entries 22–42, Table 1) displayed no levels of inhibition at the maximal concentration used in the assay. By contrast, members of the aliphatic and poly-functional, heteroaromatic libraries (entries 1–21 and 43–70, Table 1) displayed varying degrees of activity. The optimal length and branching for an aliphatic side chain appears to be when $R = C_4H_9$ as this is the common element of four of the five aliphatic analogues the displayed inhibition in Table 1. Incorporation of a pyridyl side chain, promoted inhibition or death of the nauplii in five of the

Table 1. Summary of piperazine-2,5-dione derivatives[a]

No.		Yield[b] (%)	ES–MS[c] Observed mass ion (MW)	Inhibition level[d] (μg/mL)[e] or LD_{50} (μg/mL)[e]	No.		Yield (%)	ES–MS[c] Observed mass ion (MW)	Inhibition level[d] (μg/mL)[e] or LD_{50} (μg/mL)[e]
1	**2aa**[18]	15	165 (166)	100	36	**2il**	26 **2ll**	357[f] (340)	
2	**2ab**	46	181* (180)	> 100	37	**2jj**[12]	76	379 (380)	> 100†
3	**2ac**	0			38	**2jk**	54	379 (380)	> 100†
4	**2ad**[19]	17	191 (192)	> 100†	39	**2jl**	26	368 (369)	> 100†
5	**2ae**	21	221 (222)	> 100	40	**2kk**[20,21]	68	379 (380)	> 100†
6	**2af**[15]	14	277[g] (254)	> 100†	41	**2kl**	56	368 (369)	> 100†
7	**2bb**	23	193 (194)	> 100†	42	**2ll**[22]	55	357 (358)	> 100†
8	**2bc**	10	207 (208)	> 100	43	**2mm**	64	367 (368)	> 100†
9	**2bd**[15]	24	205 (206)	> 100	44	**2mn**	38	331* (330)	> 100†
10	**2be**	51	235 (236)	100	45	**2mo**	95	336* (335)	> 100†
11	**2bf**[15]	18	267 (268)	> 100†	46	**2mp**	69	388 (389)	> 100†
12	**2cc**	83	223* (222)	> 100	47	**2mq**	87	388 (389)	> 100†
13	**2cd**[15]	41	219 (220)	> 100	48	**2mr**	13	426[h] (419)	> 100†
14	**2ce**	88	249 (250)	> 100	49	**2ms**	41	418 (419)	100‡
15	**2cf**[15]	51	281 (282)	> 100	50	**2nn**[23]	51	293* (292)	100†
16	**2dd**[16]	6	217 (218)	> 100†	51	**2no**	56	298* (297)	100‡
17	**2de**	47	247 (248)	100	52	**2np**	64	350 (351)	**60**†
18	**2df**	0			53	**2nq**	73	352* (351)	**36**†
19	**2ee**	36	285[i] (278)	100†	54	**2nr**	44	382* (381)	> 100
20	**2ef**	35 **1f**	255[j] (310)		55	**2ns**	50	380 (381)	100‡
21	**2ff**[20]	30 **1f**	269[k] (342)		56	**2oo**[24]	51	301 (302)	> 100‡
22	**2gg**[12,25]	82	289 (290)	> 100†	57	**2op**	34	355 (356)	> 100†
23	**2gh**[26,27]	49	319 (320)	> 100	58	**2oq**	93	357* (356)	**18**†
24	**2gi**	48	329[l] (306)	> 100	59	**2or**	35	387* (386)	> 100‡
25	**2gj**[12]	76	334 (335)	> 100‡	60	**2os**	90	385 (386)	> 100†
26	**2gk**	95	334 (335)	> 100†	61	**2pp**[28]	77	409 (410)	> 100†
27	**2gl**	75	323 (324)	> 100	62	**2pq**	60	409 (410)	> 100‡
28	**2hh**[29]	81	349 (350)	> 100†	63	**2pr**	24	439 (440)	> 100†
29	**2hi**	35 **2hh**	351[m] (336)		64	**2ps**	48	439 (440)	100†
30	**2hj**	83	364 (365)	> 100‡	65	**2qq**[30]	66	409 (410)	> 100†
31	**2hk**	83	364 (365)	> 100†	66	**2qr**	31	441* (440)	100‡
32	**2hl**	65	353 (354)	> 100†	67	**2qs**	60	439 (440)	100‡
33	**2ii**	6 **1i**	259[n] (322)		68	**2rr**	95	471* (470)	100†
34	**2ij**	17 **2jj**	379[o] (351)		69	**2rs**	35	469 (470)	> 100†
35	**2ik**	50 **2kk**	379[p] (351)		70	**2ss**[31]	85	469 (470)	> 100†

[a]**a** = CH_3, **b** = CH_2CH_3, **c** = $CH(CH_3)_2$, **d** = CH=CHCH$_3$, **e** = $CH_2CH_2CH_2CH_2CH_3$, **f** = CH=CHPh, **g** = Ph, **h** = *p*-$CH_3OC_6H_4$, **i** = *p*-HOC_6H_4, **j** = *p*-$O_2NC_6H_4$, **k** = *o*-$O_2NC_6H_4$, **l** = *p*-ClC_6H_4, **m** = 3-Indolyl, **n** = 2-pyridyl, **o** = thienyl, **p** = 3,4-$(CH_3O)_2$–C_6H_4, **q** = 3,5-$(CH_3O)_2$–C_6H_4, **r** = 2,4,6-$(CH_3O)_3$–C_6H_4, **s** = 3,4,5-$(CH_3O)_3$–C_6H_4.

[b]Overall yields from diacetylglycine are given.

[c]Mass ions are generally [M−H] and obtained in the negative mode. * indicates that mass ion is [M + H] obtained in positive mode.

[d]Lowest value at which severe inhibition of nauplii movement was observed.

[e]† or ‡ indicates that the compound was partially insoluble at 100 μg/mL or at 10 μg/mL, respectively.

[f][M−H] for **2ll**.

[g][M + Na].

[h][M + Li].

[i][M + Li].

[j][M−CH_3] 255, calculated mass for **1f** is 270.

[k][M−H] 269, calculated mass for **1f** is 270.

[l][M + Na].

[m][M + H] for **2hh**.

[n][M−H] 259, calculated mass for **1i** is 260.

[o][M−H] for **2jj**.

[p][M−H] for **2kk**.

six analogues bearing this group. Polyether functionality also gave rise to inhibition or death, however, no discernable pattern was present in these analogues, as to the position or degree of substitution of the aromatic ring. Although the 3,5-dimethoxy group was present in four active analogues, combination with itself (**2qq**, entry 65, Table 1) provided no activity and combination with the thienyl group, which did not display activity in other analogues within the library provided surprisingly the most active analogue in the current library, **2oq**. The identification of **2oq** as the most active analogue serves to illustrate the power of combinatorial chemistry to identify nonrationally designed lead compounds that may have potential therapeutic value.

Conclusions

In summary, we have developed a strategy to quickly generate a library of piperazine-2,5-dione with high purity by solution-phase parallel chemistry. The practical ease with which piperazine-2,5-dione libraries can be prepared is worth stressing. The majority of reported combinatorial library synthesis involves specialized

solid supported methodologies, and equipment. In contrast the use of solution-phase synthesis described herein would require little specialized knowledge or financial investment. While the three sets of compounds described above are only of small size, their purpose is purely illustrative. The range of functionalised sidechains included in the piperazine-2,5-dione core for this small library demonstrates the versatility and scope of this procedure. Given the range of aldehydes used, one can readily visualize a diverse range of potential library substrates and products. More importantly the strategy will be applicable to the synthesis of other biologically active piperazine-2,5-diones, such as calpain inhibitors,[32] which bear N-substitutents and saturation at C2 and 6. Through the screening procedure we have identified key side chains that are potential pharmacophores for the generation of the next library of compounds. We have identified three piperazine-2,5-dione derivatives, **2np**, **2nq** and **2oq** that are toxic compounds towards brine-shrimp and thus potential lead compounds for more specific cytotoxicity assays. We are currently pursing the combinatorial synthesis of other analogues based on the current results. Further screening of analogues against specific assays to eliminate toxic analogues that are not antitumor compounds and identify which analogues are antitumor compounds will be carried out in the future.

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14. Examples of ^{1}H NMR data for compounds in Table 1. 3,6-Bis(pentylidene)-piperazine-2,5-dione 2ee: ^{1}H NMR (200 MHz, $CDCl_3$, TFA-*d*): 0.90–0.97 (m, 6H, 2×CH_3), 1.25–1.40 (m, 8H, 4×CH_2), 2.28–3.20 (m, 4H, 2×CH_2), 6.42 (t, $J_{CH,CH2}$ = 7.5 Hz, 2×CH), NH not observed. 3-(3,4-dimethoxy-benzylidene)-6-(3,4,5-trimethoxy-benzylidene)piperazine-2,5-dione 2ps: ^{1}H NMR (200 MHz, $CDCl_3$, TFA-*d*): 3.89 (s, 9H, 3×OCH_3), 3.92 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 6.60 (s, 2H, *o*-$C_6H_2(OCH_3)_3$), 6.82–7.10 (m, 5H, C=C<u>H</u>$C_6H_3(OCH_3)_2$, C=C<u>H</u>$C_6H_2(OCH_3)_3$, *o*-$C_6H_3(OCH_3)_2$, *m*-$C_6H_3(OCH_3)_2$), 8.2 (br s, $W_{h/2}$ 8 Hz, 2H, 2×NH).
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